RAPID MASS MULTIPLICATION OF TWO OVER-EXPLOITED ORCHIDS OF NORTH-EASTERN INDIA

BY

MISS. AOLEMLA PONGENER

A THESIS SUBMITTED

IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BOTANY

OF

DEPARTMENT OF BOTANY

NAGALAND UNIVERSITY, LUMAMI,

NAGALAND, INDIA

2010

"RAPID MASS MULTIPLICATION OF TWO OVER EXPLOITED ORCHIDS OF NORTH-EASTERN INDIA"

BY

MISS. AOLEMLA PONGENER

A THESIS SUBMITTED

IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BOTANY

OF

DEPARTMENT OF BOTANY

NAGALAND UNIVERSITY, LUMAMI,

NAGALAND, INDIA

2010

CONTENTS

****

NAGALAND UNIVERSITY

June 2010

DECLARATION

I, ***AOLEMLA PONGENER***, bearing registration number 278/2007 (November 24, 2006), hereby, declare that the subject matter of my thesis entitled **"RAPID MASS MULTIPLICATION OF TWO OVER-EXPLOITED ORCHIDS OF NORTH-EASTERN INDIA"** is the record of work done by me, and that the contents of this thesis did not form the basis for award of any degree to me or to anybody else to the best of my knowledge. The thesis has not been submitted by me for any research degree in any other University/Institute.

This thesis is being submitted to the Nagaland University for the degree of Doctor of Philosophy in Botany.

Aolem 28/06/10

(Aolemla Pongener)
Candidate

Ch. Ha Ranjan Deb 28/06/10

(Dr. Chitta Ranjan Deb)
Supervisor

Dr. Chitta Ranjan Deb
Faculty, Deptt of Botany
Nagaland University, HQ : Lumami
Mokokchung-798601, India

28/06/10

(Prof. N. S. Jamir)
Head of the Department

Head
Department of Botany
Nagaland University
Hqs. Lumami.

Acknowledgement

No creation in this world is a solo effort and neither is this work. My endeavors to grasp the target would never have been fruitful unless I was blessed with so many adept jewels of my field. Their help is ineffable, yet I would be very remiss if I did not render my appreciation to them.

Dr. Chitta Ranjan Deb, Faculty, Department of Botany, Nagaland University, Lumami, his indefatigable guidance and inspiration, and his unwavering faith in me, throughout my research period, for which I am in awe of, will remain a hard earned treasure. I am eternally grateful for his esteemed and meticulous superintendence.

Prof. K. Kannan, Hon'ble Vice-Chancellor and *Prof. P.Lal*, Dean, School of Sciences, N.U., for their encouraging enthusiasms during the pursuit of my research work.

Prof. N.S. Jamir, Head, Department of Botany, N.U., for his magnanimous motivation and tendering the requisite resources without which my research would not have proceeded.

Dr. S.K. Chaturvedi, Dr. (Mrs.) Talijungla, Dr. (Mrs.) Limasenla, Dr. Neizo Puro, Mr. Sanjoy Kumar, Faculties, Department of Botany, N.U., for their unprejudiced encouragement and valuable suggestions during the course of the present study.

Mr. Rongpangzulu and Dr. Bendangmenla, to whom I am indebted for their priceless contributions and unending encouragements.

Mrs. Madhabi Deb, for her selfless love and immeasurable assistance in various forms.

My friends especially *Dr. Temjensangba, Mr. Sungkumlong, Mr. Bendangchuchang, Mr. Imliyanger, Ms. Arenmongla*, for their invaluable input and endless support.

My sister, my brothers and my loved ones, for their incredible help and advice.

Last but not the least, *'My Mom and Dad'*, whom I owe my life to, for their thrilled and sustained devotion, blessings and constant prayers, which have helped me to strife and conclude my quest.

Aolem 28/06/10

(AOLEMLA PONGENER)

Lists of Figures

Figure 8: Different stages of *in* vitro morphogenetic response of nodal explants. **(a-b:** ***C. aloifolium*** **and c-d:** ***C. iridioides*****). a.** Sprouting of new shoot buds from the cultured nodes, **b**. Multiple shoots developed from the nodal segment; **c.** Sprouting of new shoot buds from the cultured nodal explants of *C. iridioides* and **d.** Multiple shoots/buds formed from the cultured nodal explants.

Figure 9: Plantlets regeneration and multiple shoots/buds formation of *C. aloifolium* on regeneration medium containing different substratums. **a.** Multiple shoots/buds/PLBs formed on agar gelled regeneration medium; **b.** Multiple shoots/buds/PLBs formed on regeneration medium containing foam as substratum; **c.** Multiple shoots/buds/PLBs formed on regeneration medium containing coconut coir as substratum.

Figure 10: Plantlets regeneration and multiple shoots/buds formation of *C. iridioides* on regeneration medium containing different substratums. **a.** Multiple shoots/buds/PLBs formed on agar gelled regeneration medium; **b.** Multiple shoots/buds/PLBs formed on regeneration medium containing foam as substratum; **c.** Multiple shoots/buds/PLBs formed on regeneration medium containing coconut coir as substratum.

Figure 11: Effects of different substratums on regenerations and mass multiplication of *C. aloifolium* plantlets *(with MS medium containing 3% sucrose, 3 BA μM).*

Figure 12: Effects of different substratums on regenerations and mass multiplication of *C. iridioides* plantlets *(with MS medium containing 3% sucrose, 3 + 6 μM NAA + BA respectively in combination).*

Figure 13: Hardening of regenerated plants and transplanting to community potting mix. **a-b:** ***C. aloifolium*****; a.** One of the well rooted regenerated plant in hardening condition, **b.** One of the well hardened plants transplanted to community potting mix; **c-d:** ***C. iridioides*****: c.** A rooted plant in hardening condition; **d.** Hardened plants transferred to community potting mix.

Lists of Tables

Chapter-1

Introduction

Biodiversity is the variety and differences among living organisms from all sources, including terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part. This includes genetic diversity within the species and of ecosystems. Thus, in essence, biodiversity represents all life. Biological diversity is the very basis of human survival and economic well-being as it provides food, clothing, shelter, medicine, biomass, energy and industrial raw materials, and yet there remains a great deal or waiting to be discovered for human use. It is estimated that the species diversity living in our planet is about 5 to 50 million, out of which only 1,435,662 species have been described so far (Wilson, 1988). But, the rich biodiversity of the planet is under siege due to various factors. The human population has witnessed a three-fold increase in the last century and the rate of fossil fuel consumption has increased by 12-fold during the period, by which the carrying capacity of earth would saturate by the middle of this 21st century (Myers, 1990). According to IUCN (IUCN. 2000, *Red List of Threatened Species,* Switzerland: The World Conservation Union), plant species are declining in south and central America, Central and West Africa and Southeast Asia.

Malaysia has the most threatened species (681) followed by Indonesia (384), and so on. Globally, the number of threatened plants listed is 5,611, but this is based on an assessment of only 4% of the world's described plants, which suggest that the percentage of threatened plants may be much higher. The recent report of IUCN brings out a list of 34 biodiversity hotspot regions of the world which indicates an alarming situation the world is faced with, in terms of biodiversity resource *vis-a-vis* future of mankind. Biodiversity hotspots are geographical regions which are extremely rich in species, have high endemism, and are under constant threat. The 34 biodiversity hotspots by region are: 4 in North and Central America (*California floristic province, Caribbean Island, Madrean pine-oak woodlands, Meso-America*); 5 in South America (*Atlantic Forest, Cerrado, Chilean Winter Rainfall-Valdivian Forest, Tumbes-Choco-Magdalena, Tropical Andes*); 4 in Europe and Central Asia (*Caucasus, Irano-Antolian, Mediterranean, Mountains of Central Asia*); 9 in Africa (*Cape Floristic Region, Coastal forest of eastern Africa, Eastern Afromontane, Guinean Forest of West Africa, Horn of Africa, Coastal Forest of Eastern Africa, Madagascar and the Indian Ocean Islands, Maputaland-Pondoland-Albany, Succuent Karoo*); 13 in Asia-Pacific (*East Melanesian Island, Himalaya, Indo-Burma, Japan, Mountains of Southwest China, New Caledonia, New Zealand, Philippines, Polynesia-Micronesia, Southwest Australia, Sundaland, Wallacea, Western Ghats and Sri Lanka*).

India is one of the 17 mega biodiversity countries, and has 26 recognized endemic centres that accounts for nearly a third of the flowering plants, though it constitute only 2.4% of land mass. Also, it is a host of 3 biodiversity hotspots viz: Himalayas, Indo-Burma, and Western Ghats and Sri Lanka. It is not only rich in biological diversity but is also an important centre of origin of agri-biodiversity. The endemism of Indian biodiversity is impressively high with about 33% of the country's

recorded flora being endemic to the country and are concentrated mainly in the North-East India, Western Ghats, North-West Himalayas and Andaman and Nicobar islands. India has a total of 89,451 animal species accounting for 7.31% of the faunal species in the world and the flora accounts for 10.78% of the global total (MoEF 1999). But according to State of Forest report 1999 (FSI, 2000), the forest cover in India is losing at an alarming rate coupled with various factors, which poses greater threat to the rich biodiversity of the country. The main causes of habitat loss are agricultural activities, extraction (including mining, logging and harvesting) and unplanned developmental works. However, according to Wood *et al.,* (2000) the underlying causes of biodiversity loss are poverty, macroeconomic policies, international trade factors, policy failures, poor environmental law/weak enforcement, unsustainable development projects and lack of local control over resources.

North-East India is a centre of megabiodiversity and is equally rich in flora and fauna and contains more than one-third of the country's total biodiversity. It lies between 22° 9′ - 29° 6′ N latitude and 89° 7′ E longitude. North-East India is the most important floristic region owing to its rich biodiversity and inhabits some botanic rarities. It is known for its diverse and most extensive lush forest cover and species composition, but is one of the major regions facing severe deforestation. The region is one of the 18 hottest hotspots of the world, having at least 7, 500 flowering plants out of which 750-800 are orchids, 58 bamboos, 64 citrus, 28 conifers, 500 mosses, 700 ferns and 728 lichen species. The region is considered a meeting region of temperate east Himalayan flora, paleo-arctic flora of Tibetan highland and wet evergreen flora of South East Asia and Yunnan, forming a bowl of biodiversity. The region host a number of botanical curiosities like *Sapria himalayana, Nepenthes khasiana* and saprophytic orchids like species of *Epipogium and Galeola* and primitive angiospermic plants like *Exbucklandia,*

Manglietia, Holboellia, etc. The rich presence of ancient plants like *Magnolia, Michelia, Camellia, Rhododendron,* orchids etc. in the region indicates that evolutionary development in wild and cultivated plants are continuously taking place (Chowdhery, 2001). Takhtajan (1969) named the region "*The cradle of flowering plants*" which is one of the richest and most interesting floristic regions of India, with orchids forming a prominent feature of the vegetation.

The region of North-East India is blessed with almost all types of vegetation and has a number of 'sacred groves or forest'. It is estimated that out of 1229 species of orchids known from India, about 750 to 800 species are found in North-East region of the country (Chowdhery, 1998; Deb *et al.*, 2003; Deb and Imchen, 2008; Hynniewta *et al.*, 2000; Kataki, 1986; King and Pantling, 1898; Kumar and Manilal, 1994; Pradhan, 1979). A comparative analysis of distribution of orchid species within the region shows that maximum diversity is found in Arunachal Pradesh followed by Sikkim and the lowest in Tripura. The region has the highest number of monotypic orchid genera while large number of saprophytic orchid species belonging to the genera *Aphyllorchis, Cymbidium, Epipogium, Eulophia, Galeola, Gastrodia, Stereosandra*, etc are also present. On the other hand, due to wide altitudinal variation and topographical features supported by favourable climatic conditions, the state is endowed with a rich floristic biodiversity including huge number of orchid species. The habitats of orchids are classified on the basis of different vegetation and forest types. They are: tropical moist evergreen and deciduous forest type (100- 1000 m), subtropical evergreen and semi evergreen forest type (1000- 2000 m), temperate and sub temperate forest type (2000-3500 m) and alpine zone (3500- 5000 m).

The family Orchidaceae is one of the largest and the most evolved family among the flowering plants with a worldwide distribution comprising about 25,000-35,000

species in some 800 genera (Chowdhery, 2001; Deb *et al.*, 2003; Deb and Imchen, 2008). More new species are being added every year. Orchids are distributed all over the world excepting Antarctica and are found in almost every colour except black. In Australia, two species i.e., *Rhizanthella gardneri* and *R. slateri* are found in underground condition and are extremely rare. They exhibit an incredible range of diversity in shape, size, and colour of their flowers, and are highly valued for their beauty. These wondrous and beautiful plants have been attracting floriculturists since time immemorial and have led to '*Orchid Mania*' throughout the world. They command a high market value due to their beautiful and long-lasting flowers. In addition to their commercial value, orchids are of considerable importance in medicines, food, and perfumes, while some are reported to have antibacterial activity (Deb *et al.*, 2009).

Based on their varying habits, orchids are classified into saprophytes (without leaves), terrestrial and epiphytes (stem/pseudobulb with leaves). Majority of the orchids are epiphytes and generally found perched on tree trunks. Some grow as terrestrial on land, as lithophytes on rock/stones and as saprophytes on decaying organic materials. Roots help them in anchoring with the substratum of their habitat. The smallest orchid measures only 1 (one) mm across (*Bulbophyllum globuliforme*), while *Vanilla* species can climb the tallest tree in the forest and can be up to 20 m long whereas, *Grammatophyllum speciosum* is the biggest orchid in the world. Orchids are differentiated not only by their flowers but also by their leaves and roots. Orchids are further divided into two types on the basis of vegetative structure and its growth: ***Monopodial orchids*** which do not have rhizomes or pseudobulbs but grow from single vegetative apex continuously season after season eg: *Aerides, Rhynchostylis, and Vanda,* and ***Sympodial orchids*** which have a number of vegetative apices situated in the rhizomes, e.g.: *Coelogyne, Dendrobium, Bulbophyllum*. The rhizomes/bulb/pseudobulbs

act as reserved organ and help the orchids to combat the extreme drought conditions faced by epiphytic orchids. Orchids stand distinct in having velamenous roots; zygomorphic flowers with well-developed gynostegium, compound pollen, elaborate perianth and resupinate ovaries; and microscopic and non-endospermic seeds with undifferentiated (reduced) embryos. The orchids are adapted to insect pollination; their flowers flaunt a variety of temptations, i.e. bright colours, a safe landing platform in the form of labellum (lip), a nourishing drink, tantalizing odours and even sex to the pollinators (insects).

Orchids are known to mankind for the last several centuries for their beautiful and attractive flowers and as medicinal plants. It was Theophrastus, a Greek Philosopher, who has mentioned in his writing "Enquiry into Plants'. '*For the Chinese*' who have been growing orchids for the last 500 years, it is the symbol of Scholar–unassuming, enduring and ascetic. It also stood as a symbol of love, beauty, grace, nobility and elegance in a woman. "*Paint bamboo when you are angry, orchids when you are happy*" is a well-known Chinese saying. Orchids have adapted themselves to extremes of the environmental conditions producing thereby great variations in vegetative forms and one may often find it difficult to identify them as orchids if they are not flowering. Many orchid flowers resemble in shape of a slipper (*Paphiopedilum, Cypripedium*), dancing girl (*Oncidium, Renanthera*), moth (*Phalaenopsis*), spider (*Brassia*), scorpion (*Arachnis*), bee (*Ophrys*), pineapple orchid (*Dendrobium densiflorum*), etc. Orchids are popular worldwide due to their marvellous flower architecture and the spectrum of colours, and comprise one of the most successful groups of plants- the Orchidaceae. (370-285 BC) (Chowdhery, 1998).

The North-East region of India is rich in biodiversity, which has played an important role in the economy of the region from ancient times. Unfortunately the plant

genetic resources of the region in general and orchid diversity in particular are fast depleting due to indiscriminate felling of forest trees including ground vegetations for 'slash and burn/shifting cultivation' together with ruthless exploitation of plants for trade and unplanned human activities. In the recent years, extinction has been the destiny of a great number of plant species including several unique and irreplaceable varieties, while many await a similar fate. Some of the rare and endemic, threatened, endangered orchids of the region are *Arachnis flos-aeris, A. labrosa, Aerides odorata, Anoectochilus crispus, Bulbophyllum rotschialdianum, Calanthe ciciliae, Ceratostylis himalaica, Cleisostoma appendicutatum, C. filiforme, C. racimeferum, Coelogyne hitendrae, C. suaveolens, C. griffithii,* ***Cymbidium aloifolium, Cymbidium iridioides,*** *C. tigrinum, Dendrobium aggretum, D. chrysotoxum, D. densiflorum, D. devonianum, D. moschatum, D. nobile, D. williamsonii, Eria alba, Liparis bituberculata, Oberonia clarkii, O. denculata, O, orbicularis, Panisia apiculata, Paphiopedelium hirsutissimum, Peristylus mannii, Pholidota griffithii, Renanthera imschootiana, Taenia latifolia, T. viridi-fusca, Vanda bicolor, V. coerulea,* and many more.

Orchids are an important ornamental crop in floriculture industry due to their beautiful foliage, colourful and fragrant flowers of varying shapes, and long vase life of cut flowers. The amenability of these plants to hybridization has been successfully exploited by man to raise novel and striking hybrids in his horticultural pursuits (Vij, 2002, Deb, 2010). This fascinating feature has placed them at the top most position in aesthetic world. This group of plants is highly valued both in the national as well as in the international markets. However, unlike the south Asian countries, India has not been able to make inroads into this multibillion dollar business despite rich natural wealth of orchid diversity (Kumar and Manilal, 1994). So, commercial orchid growing is primarily in the hands of hobbyist and nurserymen, who collect orchids from naturally grown

population, to meet their national and international commitments, adding to conservation related problems (Chadha, 1992).

Although, one of the largest families among flowering plants, the orchids are also probably among the most seriously threatened group of plants. Their vulnerability stems from two factors: the first being their highly specialized nature of germination and growth in association with a specific fungus and pollinator insects, and second being the attractive and beautiful flower of many species, making them so-sought after by man. In recent years though biotechnological means have been adopted for their multiplication in mass scale and a ban has been imposed for their wild collection for trade under *Convention on International Trades in Endangered Species* (CITES) regulations, through wild life conservation laws, still there are activities of collections from the wild. In developing countries like India, shifting cultivation and continual expansion of agricultural land coupled with deforestation for developmental activities have been a major threat to these plants. Each orchid species is adapted to life in a specialized environment. Because of their specialized requirements many orchids are very restricted in distribution and endemism is high in many cases (Hegde and Sinha, 2002). Habitat destruction and disturbances coupled with lack of ecological awareness of people in general have driven some of the orchids from their natural niches to near extinction. Destruction and fragmentation of forest causes decrease in pollinator population. This results in the low frequency of pollinators visiting the orchid flowers. Nearly 98 per cent of flowering individuals fail to set fruits under natural conditions due to lack of pollinators (Calvo, 1993). Mass propagation using conventional and tissue culture techniques thus seem to be the only strategy to commercialize orchids and conserve their natural populations from collection pressures (Vij, 2002).

The orchid seeds are exceedingly small and non-endospermous with undifferentiated embryos and produced in large numbers and their germination in nature depends upon a suitable association with a mycorrhizal fungus to provide an essential physico-chemical stimulus for initiating germination (Harley, 1959). Ever since Knudson (1922) demonstrated the possibility of bypassing the fungal requirement of orchid seeds during germination *in vitro*, asymbiotic seed germination has been accepted as an important tool for propagating orchids (Arditti *et al.*, 1982). The orchids are primarily sexual but they also reproduce and propagate by vegetative means as well through seeds. The rate of vegetative propagation (i.e. keikis, back-bulbs, division of shoots etc.) is very slow in many orchid species and seed germination in nature is also very poor (~0.2-0.3%) because of their poorly organized and lack of an appropriate metabolic machinery to utilize their own lipidaceous food reserves, and require a fungal stimulus for germination in nature. The asymbiotic germination potential of fertilized ovules (seeds) has been positively tested in several commercially viable and or threatened Indian taxa (Vij, 2002). But, not all the orchids need the same nutrient composition and response of orchid seeds to physio-chemical factors differs from species to species.

Plant Tissue Culture and Mass Multiplication and Conservation of Rare and Threatened Orchids

Orchids which were earlier thought to be parasites growing on trees are in fact the most advanced group of flowering plants. The orchids are propagated by vegetative means as well through seeds. However, orchid seeds due to microscopic size i.e lack of endosperm and require a special fungal association (mycorrhiza) to germinate in nature. The rate of seed germination, therefore, is very poor, i.e., 0.2-0.3% in nature (Sungkumlong and Deb, 2008). The mycorrhizal association is believed to help in the

carbohydrate/auxin/vitamin transport. Knudson (1922) for the first time demonstrated the possibility of bypassing the fungal requirements during germination of *Cattleya* seeds *in vitro* with the supply of appropriate organic carbon in the medium, while Tsuchiya (1954) discussed the possibility of germinating orchid seeds from immature pods. The discovery of these two techniques led to the development of 'green pod culture' that enabled to rescue hybrid embryos from desired mating (Sagawa, 1963). However, it calls for devising protocols for rapid cloning for exploitation of elite hybrids. *In vitro* cloning of *Phalaenopsis* using uni-nodal floral stock cuttings was developed by Rotor (1949), whereas Thomale (1957) successfully cultured the shoot tips of *Orchis maculata,* but the possibility of using aerial roots for micropropagation was first suggested by Beechey (1970). Morel (1960) is credited for mass propagation of virus free *Cymbidium* clones from apical shoot meristem on Knudson 'C' medium. Shoot tips remain the most commonly used explants for micropropagating *cymbidiums* and other sympodial orchids but their utility is limited in monopodials as it involves the removal of the only growing apex, which endangers the survival of the mother stock. Endeavors should, therefore, be made toward exploring an alternative but equally effective technique whose excision will not be detrimental to the survival of the mother plant. Different workers have reported regeneration of plants in cultures using different explant sources like shoots, roots, seeds, axillary buds, pseudobulbs, leaf (Deb and Imchen, 2010; Deb and Temjensangba, 2005, 2007a, b; Deb and Sungkumlong, 2010; George and Ravishankar, 1997; Li and Xu, 2009; Nayak *et al.*, 1997; Prasad *et al.*, 2000; Sinha and Hegde, 1997; Temjensangba and Deb, 2005a, b, c, 2006; Vij and Pathak, 1999; Vij *et al.*, 2000) and through callus induction and somatic embryogenesis (Ishii *et al.*, 1998). Biotechnological tools like plant tissue culture techniques have thus opened new possibilities in conservation of threatened/endangered plants.

Many orchid species have been propagated successfully through this technique particularly the threatened orchid species and re-introduced into the wild ameliorating their status in nature. Different explants sources like seeds, leaf, rhizome, roots, inflorescence, etc have been used to propagate *in vitro* in various parts of the world for conservation programme. Following are some of the works done by various workers: *Aerides multiflora* Roxb. (seeds- Katiyar *et al.,* 1987; foliar segment- Vij and Pathak, 1990; aerial roots- Vij- 1993); *Arachnis labrosa* (seeds- Temjensangba and Deb, 2005a; foliar segments- Deb and Temjensangba, 2007a; aerial roots- Deb and Temjensangba, 2006a); *Cleisostoma racemiferum* (seeds and leaf- Temjensangba and Deb, 2005b, c, 2006; aerial roots- Deb and Temjensangba, 2005); *Coelogyne porrecta* Lindl. (seeds- Abdul and Hairani, 1990); *C. suaveolens* Lindl. (seeds- Sungkumlong and Deb, 2008, leaf – Deb and Sungkumlong, 2010); *Cymbidium elegans* Lindl. (seeds- Raghuvanshi *et al.*, 1991); *Dendrobium chrysanthum* Wall. ex Lindl. (seeds- Raghuvanshi *et al.*, 1986); *D. fimbriatum* var. *oculatum* Hk. f. (D.Don) (seeds- Devi *et al.*, 1990); *D. nobile* Lidl. (seeds- Raghuvanshi *et al.*, 1986); *D. primulinum* Lindl. (seed- Deb and Sungkumlong, 2009); *Eulophia alta* (L.) Fawcett & Rendle (seed- Johnson *et al.,* 2007); *E. hormusjii* Duth. (rhizome segments- Vij *et al.*, 1989); *Haemaria discolor* (Mandarin: Xue-ye-lan or Cai-ye-lan) (seeds- Shiau *et al.,* 2005); *Luisia teretifolia* Gaud. (foliar segments- Vij and Pathak, 1990); *Malaxis khasiana* Soland ex. Swartz (seeds- Deb and Temjensangba, 2006b); *Rhynchostylis gigantia* (immature seeds- Li and Xu, 2009); *Rhynchostylis retusa* (L.) Bl. (seeds- Nath *et al.*, 1991; aerial roots- Chaturvedi and Sharma, 1986; Sood and Vij, 1986; foliar segments- Vij and Pathak, 1990); *Taenia latifolia* Lindl. (seed- Deb and Sungkumlong, 2008; pseudobulb- Sungkumlong and Deb, 2009, leaf- Deb and Sungkumlong, 2010); *Vanda cristata* Lindl. (foliar segments- Vij and Pathak, 1990); *V.*

testaceae (Lindl.) Reichb. f. (foliar segments- Vij and Pathak, 1990); *Vanda* Kasem's Delight 'Tom Boykin (aerial roots- Vij and Sharma, 1997).

A wide range of endangered plants including orchids have now been successfully propagated using *in vitro* techniques. There are many reports on *in vitro* multiplication of different types of orchids. Different workers have reported regeneration of plants in cultures using different explant sources like shoots, roots, seeds, axillary buds, pseudobulbs and leaves.

Seed/Embryo Culture

The technique is variously referred to as ovule/embryo/green pod/green fruit culture (Sagawa, 1963), which ensures better germination frequency and favours the production of virus free seedlings at a faster rate. Asymbiotic/non-symbiotic seed germination is the most common approach used in the propagation of tropical orchids, which tend to be easier to grow than their temperate relatives. The media used for asymbiotic germination are more complex than that for symbiotic germination, as all organic and inorganic nutrients and organic carbon source must be in a form readily available to the orchid without the intermediary fungus (Mc Kendrick, 2000). The technique involves an easy procedure for sterilization, ensures better frequency of germination, and reduces the time-lapse between pollination, sowing of seeds and production of virus free seedlings. Since all the seed/embryos are used in a single sowing in this technique, it is important to determine the harvest time of capsule or pod for getting optimal germination. The earliest stage at which the embryos can be cultured successfully varies with the orchid genotype and the local conditions. Very young ovules do not form suitable explants in orchids because the embryo sac development is a post pollination phenomenon and fertilization a prerequisite for obtaining seedlings. However as the ovules can be used for raising cultures immediately after fertilization, the

importance of information on time interval between pollination and fertilization has often been stressed (Valmayor and Sagawa, 1967). *Doritis* ovules from pollinated ovaries germinated readily after getting fertilized *in vitro* (Yasugi, 1984) suggesting that fertilization is a pre-requisite for germination. Yam and Weatherhead (1988) also noted that immature embryo germinates better than the mature ones due to their distended testa cells and metabolically awakened embryos; they also lack dormancy or inhibitory factors. *Arachnis labrosa* and *Cleisostoma racemiferum* embryos obtained between 16 and 18, and 16 weeks after pollination (WAP) respectively (Temjensangba and Deb, 2005a, c, 2006); readily germinate but their germination frequency declines sharply, when obtained from beyond this window period. Likewise, in *Satyrium nepalense, Nephalaphyllum cordifolium, Phaius tankervilliae* and *cymbidiums*, germination frequency shows sharp decline when the embryos are collected 3-4 weeks prior to fruit dehiscence. The fruit/capsule that develops prominent ridges along the valves and ceases to grow in diameter is considered a useful marker for selecting the right stage for embryo culture (Vij, 1995).

Meristem Culture

Resident Meristem: The embryo culture produces a great deal of heterozygosity in their progeny in orchids due to its out breeding characteristic. Because of this, it appears to be a disadvantageous proposition in cut-flower industry where pure lines of desired genotypes are preferred. The possibility of using excised shoot-meristem of *cymbidiums* for regenerating complete plant from *in vitro* was first demonstrated by Morel (1960), whereas Wimber (1963) formulated, described and published a procedure for the purpose. This technique of using resident meristem (shoot-tips, axillary bud) has opened new vistas in orchid micropropagation (Arditti and Ernst, 1993; Deb and Temjensangba, 2005, 2006a). Through this technique, upto 200,000 plants can be regenerated from a

single resident meristem within a year. However, it has limited utility in monopodial taxa as it involves the sacrifice of the growing tip thereby, endangering the survival of the mother plant.

Adventive meristems: The ability to use an adventive meristem is advantageous as it does not endanger the survival of mother plant. The regenerative competence or the proliferative potential has been positively tested in many orchid taxa, viz: leaf explants (Chaturvedi and Sharma, 1986; Deb and Temjensangba, 2007a; Deb and Sungkumlong, 2010; Mathews and Rao, 1985; Seeni, 1988; Seeni and Latha, 1992; Temjensangba and Deb, 2005b; Vij *et al.*, 1984; Vij and Pathak, 1988, 1990); root (Chaturvedi and Sharma, 1986; Deb and Temjensangba, 2005, 2006a; Sood and Vij, 1986; Vij, 1993; Vij and Pathak, 1988); flower stalks (Kaur and Vij, 1995; Singh and Prakash, 1984; Vij *et al.*, 1997). The source, genetic constitution and physiological age of the explants are however, some of the important factors for regeneration. The juvenile tissues from greenhouse grown plants respond better than the mature ones grown outdoors. Generally, the proliferative loci get activated in the sub-epidermal cells and soon develop into somatic embryos and or Protocorm-like-bodies (PLBs). Somatic embryogenesis is either direct or callus mediated development, and multiplication and differentiation of the PLBs is influenced by the chemical stimulus present in the nutrient pool (Seeni and Latha, 1992; Vij and Pathak, 1990).

The advantages of leaf and root segment culture are apparent for more than one reason: they are easy to obtain, easier to disinfect, and their excision does not endanger the mother plant. Furthermore, as the regeneration occurs in the dermal cells, which is cytologically more stable, mass production of genetically uniform plant from this is within the realm of reality (Vij, 2002).

Different species of orchids exhibit specific needs in respect to nutritional requirement and treatment with plant growth regulators (PGRs) for their growth and development. So, no standard media formulation can be prescribed for all the species. Most commonly employed basal media for orchid tissue culture are Knudson 'C' (1946), Mitra *et al,* (1976), Murashige and Skoog (MS) (1962), Nitsch and Nitsch (1969), Vacin and Went (1949). The use of α-Naphthalene acetic acid (NAA) and one of the cytokinins like Benzyladenine (BA) and kinetin (KN) yields a rich crop of PLBs in *Luisia trichorhiza, Satyrium nepalense, Vanda cristata* and *Vanda testaceae* leaf segment culture (Vij, 1995). Similarly, in *Rhynchostylis retusa*, a synergistic action of KN and indole 3-acetic acid (IAA) or NAA in peptone enriched medium favours enhanced production of PLBs; while yeast extract (YE) is obligatory for regeneration in *Aerides multiflorum, Papilionanthe teres* and *Satyrium nepalense* foliar cultures and peptone in those of Vanda (Vij, 2002).

Plant Tissue Culture Media and Substratum

Since its introduction as a gelling agent for microbial cultures more than 100 years ago, agar has been extensively used as gelling agent for microbial as well as plant tissue culture media (Babbar and Jain, 2006). Agar is useful for the purposes due to its stability, high clarity, non-toxic nature and resistance to its metabolism (Babbar and Jain, 2006; Henderson and Kinnersley, 1988; McLachlan, 1985). In the recent past several attempts have been made to look for some suitable substratum which can replace agar in the plant tissue culture media as well as microbial culture because of doubts about its inertness and non-toxic nature, fear of over-exploitation of its sources and above all the exorbitant price of tissue culture and bacteriological grade agar (Arnold and Ericksson, 1984; Babbar and Jain, 1998, 2006; Debergh, 1983; Jain and Babbar, 2002; Kohlenbach and Wernicke. 1978; Singha 1984; Zimmerman *et al.*, 1995). During the last two

decades, a number of substances viz. agarose (Johansson, 1988), alginates (Scheurich *et al.*, 1980), gelrite (Pasqualetto *et al.*, 1988), isubgol (Babbar and Jain, 1998), starch (Nene *et al.*, 1996; Zimmerman *et al.*, 1995) etc. have been used with reasonable success as substitutes of agar. But these are not expected to find universal acceptance, for various reasons. Alginates gel only in the presence of specific ions and therefore are not suitable substitutes of agar, while agarose is cost prohibitive. Starch is not expected to find universal acceptance because of its inferior gelling ability, poor clarity and metabolizable nature, which leads to softening of the media. Isubgol, due to its polysaccharide nature, good gelling ability, resistance to enzymatic activity, and gel clarity it has a good potential to become a universal gelling agent for plant tissue culture media. But its high melting point (~70°C) necessitates adjusting of *p*H and fast dispensing (Babbar and Jain, 2006). But use of these gelling agents does not help in substantially reducing the production costs.

Objectives of the Study

The loss of primary forest in North-Eastern part of India is primarily due to practice of primitive form of agriculture commonly called "Jhum", fragmentation of forest and unplanned developmental activities. As a result, many important plant species including orchids are under threat or on the verge of extinction before their commercial importance are being explored. Therefore, it calls for an immediate intervention and develop an alternate route to preserve the threatened and endangered plant species particularly orchids. A biotechnological tool like tissue culture technique comes in handy for successful achievement of the objectives. Plant tissue culture technique is in general a costly technique and faces difficulties in universal acceptance as a tool for commercial scale production of plants of horticultural/economic importance and *in vitro* conservation of rare and threatened taxa. To popularize the plant tissue culture technique

Figure 1: Selected orchids showing vegetative parts with flowers. **a**. *Cymbidium aloifolium* (L.); **b**. *Cymbidium iridioides* D. Don.

Figure-1

as a tool for commercial scale production of economically important plants and *in vitro* germplasm conservation, it is necessary to develop cost effective protocols.

With a view to mass multiply and conserve two rare/ and threatened orchids of North-East India and develop cost effective protocols, I had proposed to work on the following two economically important but over-exploited orchid species viz., ***Cymbidium aloifolium* (L.) Sw.** and ***Cymbidium iridioides* D. Don** of North-East India for my Ph. D. degree, with the following objectives:

I) ***Initiation of cultures from various explants like immature embryos of various developmental stages, leaves, nodal explants and roots from in vitro raised plants.***

II) ***Rapid mass multiplication of the selected species.***

III) ***Screening of some low cost substratums as alternative to agar for initiation of cultures and mass multiplication of the selected orchids with an objective to reduce the production cost.***

IV) ***Reintroduction of in vitro raised plants in their natural habitats.***

A Brief Account of the Two Selected Orchids

1. *Cymbidium aloifolium* (L.) Sw. (Orchidaceae): It is an epiphytic herb which grows on the tree trunks in huge clumps. They are mostly found in the tropical forests. Pseudobulbs are ovoid, short, 6-9 x 3-4 cm, bilaterally flattened, sheathed. Leaves many, upto 60 cm long, linear, oblong, apex obliquely bilobed. Inflorescence raceme, arises from the base of the pseudobulbs, pendulous, many flowered. Flowers 3 cm across, yellowish with purple mid rib. Sepals oblong-lanceolate. Petals linear-oblong, obtuse. Lip whitish with maroon or purplish streaks; 3-lobed, side lobes oblong, obtuse; mid lobe ovate, acute, and reflexed; 2 yellowish lamellae; column purplish. It flowers in the months of April-August and last for about 25-30 days. **(Fig. 1 a).** The flowers have good

commercial value as cut flowers. Besides floricultural importance, the entire plant is used as purgative, emetic, tonic and in treating ear-ache. Due to its multipurpose use, the plants are ruthlessly collected from its natural habitat and the population is under threat.

2. ***Cymbidium iridioides*** **D. Don (*C. giganteum* Wall. ex Lindl.) (Orchidaceae):** This is an epiphytic or lithophytic, perennial herb. Pseudobulbs 6-7 cm long, broad, sheathed. Leaves upto 60 cm long, linear, lanceolate, base sheathing. Inflorescence many flowered raceme, arched. Flowers 7-8 cm across, brown with red streaks. Sepals and petals linear-oblong. Lip yellowish brown, side lobes redish brown striped, ovate, acute; mid lobe yellowish with reddish brown transverse blotches, ovate-oblong, margins undulate; lamellae 2, terminating above the base of the mid lobe. Column yellowish brown. The species is predominantly found in North-east India. It flowers during the months of October-November. **(Fig. 1 b).** The flowers have high commercial value in cut flower industry due to its long lasting flowers. This species is under threat as a result of over-exploitation and indiscriminate destruction of forest cover to meet the demands of ever increasing populations and removal of ground vegetation and litters by forest fires for 'Jhum/Slash and Burn cultivation'.

Chapter-2

Materials and Methods

Micropropagation and other *in vitro* techniques have become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants and for conservation of genetic resources, particularly with those crops which are vegetatively propagated or have recalcitrant seeds which cannot be stored under conventional seed bank conditions (George, 1993; George and Sherrington, 1984). Likewise, *in vitro* culture is being used in an increasing number of threatened plant species. In addition to micropropagation in its strict sense, other techniques available include *in vitro* seed germination, regeneration of plants from callus cultures, dual culture with symbiotic fungi and micro grafting. The use of these techniques have allowed the propagation of many rare, threatened and endangered plant species which prove problematic with conventional horticultural methods and particularly useful with groups of plants which are difficult to propagate using conventional techniques.

Orchids are inherently slow growers and seeds germinate poorly in nature due to their nutritional complexities. Traditionally orchids are mainly propagated through vegetative means such as keikis, back-bulbs, division of shoots etc., and through seeds.

Vegetative propagation is very slow while the seed germination in nature is very poor (~0.2-0.3%) (Sungkumlong and Deb, 2008). Their regeneration in nature is limited due to suppressed endosperm and requirement of fungal stimulus. But this constraint to commercial production has been overcome by the development of tissue culture techniques which have also opened new possibilities in conservation and commercialization of orchids (Deb and Sungkumlong, 2009, 2010; Lal *et al.*, 1988; Sungkumlong and Deb, 2008; Tandon *et al.*, 1990; Temjensangba and Deb, 2005a, 2006). Following this technique, round the year propagation of genetically uniform, disease free, fast maturing and high yielding plants are made possible and enabled to exploit the regenerative competence more effectively than the conventional method. Ever since Knudson (1922) demonstrated the ability to by-pass the fungal requirements for germination of *Cattleya* seeds/embryos *in vitro* by using appropriate level of carbohydrates in the culture medium, remarkable progress has been made in micropropagation of orchids (Arditti *et al.*, 1982; Arditti and Ernst, 1993; Bejoy *et al.*, 2004; Bhadra and Hossain, 2003; Chen *et al.*, 2004; Kalimuthu *et al.*, 2007; Kannan, 2009; Kaur and Bhutani, 2009; Kosir *et al.*, 2004; Li and Xu, 2009; Martin, 2007; Mitra *et al.*, 1976; Seeni and Latha, 1992; Sunitibala and Kishor, 2009; Temjensangba and Deb, 2005a; 2006; Vij and Pathak, 1990; Yam and Weatherhead, 1988). The immature embryos and the shoot meristem are the most commonly used explants for *in vitro* propagation of orchids. But the response of orchid seeds to physio-chemical factors differ from species to species (Arditti *et al.*, 1982; Arditti and Ernst, 1993; Bejoy *et al.*, 2004; Chen *et al.*, 2004; Mitra, 1986; Seeni and Latha, 1992; Temjensangba and Deb, 2005a, 2006; Vij and Pathak, 1990; Yam and Weatherhead, 1988).

Seed germination represents the most efficient method for orchid propagation for mass multiplication and conservation purposes (Arditti *et al.*, 1982; Stewart and

Kane, 2006; Temjensangba and Deb, 2005a). The immature seeds of various developmental stages can also germinate *in vitro* prior to reaching maturity and the technique is variously referred as ovule/embryo/green pod/green fruit culture (Bhadra and Hossain, 2003; Deb and Temjensangba, 2006b; Deb and Sungkumlong, 2009; Kannan, 2009; Li and Xu, 2009; Sagawa, 1963; Sungkumlong and Deb, 2008; Temjensangba and Deb, 2005a, 2006). However, orchid seed germination studies are often viewed as unreliable or unrealistic since little is known concerning the germination and *in vitro* seedling developmental requirements of many orchids. Compounding this difficulty, many workers (Temjensangba and Deb, 2005a, c, 2006; Deb and Sungkumlong, 2009) found that different species requires different developmental age for *in vitro* response for germination and most of the orchid species have a very short window period of developmental stage when they germinate *in vitro*. The *in vitro* germination potential of fertilized ovule/immature seeds has been positively tested in several commercially viable and/or threatened orchid taxa (Deb and Temjensangba, 2006b; Deb and Sungkumlong, 2008, 2009; Johnson *et al.*, 2007; Kannan, 2009; Stewart and Kane, 2006; Sungkumlong and Deb, 2008; Temjensangba and Deb, 2005a, 2006). Non-symbiotic seed germination of orchids is greatly influenced by several factors like green pod age/developmental stage of embryos, different nutrient media with adjuvant, plant growth regulators (PGRs) etc. Apart from immature seeds/embryos other explants like aerial roots, foliar explants, nodal segments, inflorescence could be used successfully for *in vitro* propagation of orchids (Deb and Temjensangba, 2005, 2007a; Deb and Sungkumlong, 2010; George and Ravishankar, 1997; Kosir *et al.*, 2004; Li and Xu, 2009; Nayak *et al.*, 1997; Prasad *et al.*, 2000; Sinha and Hegde, 1997; Vij and Pathak, 1999; Vij *et al.*, 2000).

In vitro culture of orchids is greatly influenced by several factors like explants age/ developmental stage, source of explants, nutrient media, organic carbon sources, different adjuncts, and quality and quantity of PGRs, light etc. Apart from different factors, for popularization of tissue culture technique in terms of commercial scale production of orchids, it has included the development of low cost protocols including low cost alternative substratum to agar as agar is one of the costly components in tissue culture.

Preparation of Substratums

Different types of substratum including 'agar, betel-nut coir, coconut coir, polyurethane foam (thereafter called '*foam*') and forest leaf litter' were selected for the present study. In the present study, plant tissue grade agar was used (make: Hi-media, India). 'Foam' was collected from the local market which is generally used for preparation of mattresses. Other substratums like 'betel-nut coir, coconut-coir were extracted from the dried fruits and chopped into small pieces, while, 'leaf litter' was collected from the forest floor before they are decayed. Except agar all other materials were soaked with 'Extron' (a commercial laboratory detergent, make: Merck, India) (1:100) (v/v) for about two hours followed by washing under running tap water till the water ran clear. The substratums were air dried and stored till used. The dried substratum except foam were chopped into small pieces (~0.5 cm size), while the foam was cut into disk (according to the culture vials). These substratums except agar were then autoclaved at1.05 Kg cm^{-2} pressure and 121°C for one hour before putting them in the culture vials.

Plant Materials

When working with rare and endangered species, the amount of available plant material can be very small, and this can place restrictions on the choice of methods.

Seeds are preferred to vegetative material as the source of propagation so that a wider genetic base can be maintained. However, in some species, seed is not readily available and therefore vegetative material has to be used. Success to a great extent depends on the selection of the right explants, physiological age, media composition, exogenous growth regulators and culture conditions.

Seeds: The immature seeds/green pods of different developmental ages of *Cymbidium aloifolium* [7-12 months after pollination (MAP) at one month interval] and *Cymbidium iridioides* (6-16 MAP at two months interval) were collected from the garden. These green pods were used for the experimental purpose.

Leaf: For both the species, foliar explants from *in vitro* grown plantlets/axenic cultures were collected and used for the present study. Foliar explants (0.5-1.5 cm size) were collected after about 5 wk of emergence just before inoculation.

Roots: Aerial roots of ~5-6 wk old were harvested from axenic cultures of both the species.

Nodal explants: Nodal explants were collected from the *in vitro* raised plantlets. The etiolated plantlets with distinct nodes were selected and the leaves were removed. The defoliated shoots were cut into segments with one or two nodes in each segment. These nodal segments were used for the initiation of culture.

Sterilization of Plant Materials

Seeds: The green pods/capsule of different developmental stages of both the species (*Cymbidium aloifolium* and *C. iridioides*) were harvested and washed thoroughly with Extron (1:100) (v/v) which were then rinsed under running tap water. The pods were surface sterilized with aqueous solution of mercuric chloride ($HgCl_2$) (0.3%) (w/v) for 5 min and subsequently rinsed 4-5 times with sterile doubled distilled water. Prior to dissection of the embryos and inoculation, the pods were dipped in ethanol (70%) (v/v)

and flamed. This operation was carried out under aseptic condition inside the laminar flow cabinet.

The aerial roots, foliar explants and nodal explants were harvested from the axenic culture and inoculated on initiation media without sterilization.

Tissue Culture

Media: For initiation of embryo/seed cultures, various media like Gamborg or (B_5) (Gamborg *et al.*, 1968), Knudson 'C' (Knudson, 1946), Mitra *et al.*, (Mitra *et al.*, 1976), Murashige and Skoog (MS) (Murashige and Skoog, 1962) and SH (Schenk and Hildebrandt, 1972) media were used. For immature seed culture of both the species, the basal media were fortified with coconut water (CW) (0-20%) (v/v), casein hydrolysate (0-200 mgl^{-2}) and different organic carbon sources such as dextrose, glucose and sucrose (0- 4%) (w/v) along with different levels of plant growth regulators (PGRs) such as α-naphthalene acetic acid (NAA) and N^6–benzyl adenine (BA) (0-9 µM for *C. aloifolium* and 0-12 µM for *C. iridioides*) singly or in combination.

Foliar explants of both *C. aloifolium* and *C. iridioides* were cultured on MS medium fortified with sucrose (0-4%) (w/v) and supplemented with different levels of PGRs such as NAA and BA (0-9 µM) singly or in combination. In case of aerial root cultures of *C. aloifolium* and *C. iridioides,* MS medium was fortified with sucrose (0-4%) (w/v), activated charcoal (AC) (0-200 mgl^{-2}) (w/v). The basal medium was further fortified with different levels of PGRs like IAA, NAA, BA, and KN (0-9 µM) singly or in combination. While, for nodal explants culture of both *C. aloifolium* and *C. iridioides*, the MS medium was enriched with sucrose 3% (w/v), and NAA and BA (0-9 µM) singly or in combination.

The *p*H of the media was adjusted to 5.6 using 0.1 N NaOH and 0.1 N HCl. The media were gelled using agar (0.8%) (w/v) before autoclaving. About 15 ml of the

medium was dispensed into each test tube (size 150 mm x 25 mm) and 30 ml into 400 ml culture bottle (diameter: 70 mm). Besides agar, different pre-processed substratums like betel-nut coir, coconut coir, foam and leaf litter were used as supporting materials to which about 10-12 ml of the prepared liquid media were dispensed in each test tube. The media were autoclaved at 1.05 Kg cm^{-2} pressures and at 121°C for 20 minutes.

Initiation of Cultures

Immature embryos/green pods: The immature seeds/embryos of different developmental stages from both the species were scoped out from the sterilized green pods under a laminar flow chamber with the help of scalpel blade and cultured on different basal media containing different substratum such as agar, betel-nut coir, coconut coir, foam and leaf litter, fortified with different levels of various adjuncts. The cultured embryos were incubated under different light conditions viz., dark, diffused (20 µmol m^{-2} s^{-1}) and full light (40 µmol m^{-2} s^{-1}) at 12/12 hr light/dark photo cycle. For each treatment 20 culture vials were maintained.

Leaf: The leaves from the *in vitro* raised plants of both the species were carefully taken out inside the laminar flow cabinet and were cultured on the nutrient medium. The intact leaves were cultured on different initiation media and in each culture vial two leaves were cultured. To determine the effect of orientation, explants were placed in a slanted (~45°) and horizontal position, also position with upside and upside down. For each treatment 20 explants were cultured.

Root segment: The aerial roots from the *in vitro* raised plants of both the species were carefully taken out inside the laminar flow cabinet and were cultured on the nutrient medium. The aerial roots from the *in vitro* source of about 0.5-1cm long were cultured on the initiation medium. In each test tube one or two root segments were cultured and about 20 root segments were used for each treatment.

Nodal explants: The processed nodal segments were cultured on the nutrient medium. In each culture vial 2 nodal segments were inoculated and for each treatment 20 segments were maintained.

The foliar explants, aerial roots and nodal explants were cultured under full light (40 μmol m^{-2} s^{-1}) condition at 12/12 hr light/dark photo cycle. All the cultures were maintained at 25±2°C and sub-cultured at 4-5 wk interval unless mentioned otherwise. The experimental design was completely randomized and all the experiments were repeated at least thrice.

The Protocorm-like bodies (PLBs)/shoot buds developed from the germinated seeds/embryos, foliar explants, aerial roots and nodal segments were maintained on the optimum initiation condition for 2 passages for further proliferation and differentiation. In the cultures with different alternative substratums, about 5 ml of fresh liquid medium was poured in the same culture vial after removing the exhausted medium with a pasture pipette.

Regeneration of Plantlets and Mass Multiplication

The PLBs/shoot buds/advanced stage PLBs (just before release of first set of leaflets) formed from the cultured immature embryos, leaf, aerial roots and nodal explants were maintained further for two passages on the optimum initiation conditions for further development and differentiation. The tiny plantlets so formed were separated from the clumps and transferred to three different basal media (Knudson 'C', Mitra *et al* and MS) containing various organic carbon sources such as dextrose, glucose and sucrose (0-4%), CW (0-20%), CH (0-0.2 gl^{-1}), different levels of plant growth regulators (PGRs) like IAA, NAA, BA and KN (0- 9 μM) singly or in combination for regeneration of plantlets and mass multiplication. Apart from agar gelled media, the cultures were also maintained on the different alternative substratums as used for germination of immature

embryos, for regeneration of plantlets and mass multiplication. In every sub-culture the shoot buds formed were separated and transferred on fresh regeneration medium. The plantlets were maintained for 2-3 passages on regeneration medium before they were transferred to the hardening condition.

Hardening of Regenerated Plants

About 5-6 cm long well-rooted plantlets (with 2-3 roots) of both the species were hardened for considerable period prior to transferring in the potting mix. The plantlets were taken out from the regeneration medium and the traces of agar were washed off with a soft brush (for cultures raised on agar gelled media) and then transferred on culture vials containing highly reduced strength of MS liquid medium ($1/10^{th}$ strength) supplemented with sucrose (1%) (w/v) devoid of any PGR, a protocol developed by Deb and Imchen (2010). In the culture vials different types of supporting materials were incorporated which includes charcoal pieces, brick pieces and chopped mosses (at 1:1 ratio). All these materials were washed thoroughly under running tap water and autoclaved at 1.05 Kg cm^{-2} pressure and 121°C for one hr before putting them in the culture vials. The cultures were maintained for 4-6 wk in 12/12 hr photoperiod with light provided at 40 µmol $m^{-2}s^{-1}$ at a temperature of 25±2°C before transferring to community potting mix (CPM).

Potting Mix and Transplantation of Regenerates

To transplant the hardened plantlets of both the orchid species (*C. aloifolium* and *C. iridioides*), the CPM was prepared by mixing different substrates like sand: brick pieces: coconut husk: charcoal pieces: decayed wood in different combinations in the ratio of 1:1:1:1 with a layer of moss. The hardened plants were transferred to CPM along with the content (substratum of the hardening medium) and were covered with holed

transparent poly bags. The potted plants were maintained in a shaded place and fed with MS liquid salt solution ($1/10^{th}$ strength) weekly for 2-3 wk. The potted plants were exposed to normal day light for about 1 hr in a day for initial one wk and subsequently increased the exposure period by 2 hr from the second wk and finally after one month the plantlets were left in the normal full day light condition which were kept for about 7-8 wk before transferring to the wild.

Chapter – 3

Results

Initiation of Cultures

Immature Embryos/Seeds

The immature seeds/embryos of various developmental stages were cultured on different basal medium containing different supplements. The green pod age, basal media composition, quantity of organic carbon, quality and quantity of PGRs were found to be crucial factors for successful culture initiation. Nodular swelling of seeds was the first sign of germination in both the species **(Table 1 & 2)**.

Effects of Basal Media on Asymbiotic Seed Germination

In ***Cymbidium aloifolium,*** the first sign of germination was observed as nodular swelling of the embryos after 25 days of culture **(Table 1)** while, in case of ***Cymbidium iridioides*** similar response was registered after 20 days of culture **(Table 2)**, which converted into healthy green PLBs after 58 days of culture in both the species **(Fig. 2a & 3a)**. Amongst the five basal media used for asymbiotic germination of seeds in the present study, MS medium was found to be superior over other four media in both the species. In *C. aloifolium* a germination rate of ~90% was achieved on MS medium followed by Mitra *et al* (70%) and Knudson 'C' (50%) media **(Table 3)**. Other two

Table 1: Effect of green pod age on *in vitro* germination of immature embryos of *C. aloifolium*

Green pod Age (MAP)	Days taken for Nodulation	PLBs formation	1st leaflets	% Germination (±SE)#	Type of response*
7	-	-	-	-	No response
8	60	90	-	30 (1.00)	Delayed germination.
9	**25**	**58**	**66**	**90 (1.50)**	**Healthy green PLBs.**
10	35	70	95	60 (1.50)	Few green PLBs.
11	35	80	95	50 (1.75)	Delayed germination.
12	8	90	130	50 (2.00)	Delayed germination.

Standard error.

* On MS medium containing sucrose (2%) (w/v), NAA and BA (3 + 6 µM respectively in combination)

Data represents the mean of three replicates.

Table 2: Effect of green pod age on *in vitro* germination of immature embryos of *C. iridioides*[*]

Green pod age (MAP)	Days taken for			Germination rate (%) (±SE)[#]	Types of response
	Nodulation	PLBs formation	1st leaflets		
6	-	-	-	-	No response
8	35	65	-	35 (±1.5)	Slight nodular swelling of embryos and fewer PLBs formed.
10	**20**	**58**	**74**	**95 (±1.5)**	**Most of the germinated embryos converted into healthy PLBs.**
12	32	67	88	85 (±2.5)	Nodular swelling followed by PLBs formation.
14	48	76	90	55 (±2.5)	Nodular swelling followed by poorer PLBs formation.
16	50	-	-	-	Only swelling but no PLBs formation.

* On MS medium containing sucrose (2%), NAA + BA (3+3 µM respectively) in combination.

Standard error.

Data represents the mean of three replicates.

Figure 2: A-symbiotic immature seed/embryo germination of *Cymbidium aloifolium* on different substratums. **a**. Culture on agar gelled medium; **b.** Seed germination on betel nut coir; **c.** On coconut coir; **d.** Cultures on foam disk; **e.** Germination on chopped forest leaf litters; **f.** Advantaged stage PLBs on germination medium ready to be transferred on regeneration medium.

Figure – 2

Figure 3: A-symbiotic immature seed/embryo germination of *Cymbidium iridioides* on different substratums. **a**. Culture on agar gelled medium; **b.** Seed germination on betel nut coir; **c.** On coconut coir; **d.** Cultures on foam disk; **e.** Germination on chopped forest leaf litters; **f.** Advantaged stage PLBs on germination medium ready to be transferred on regeneration medium.

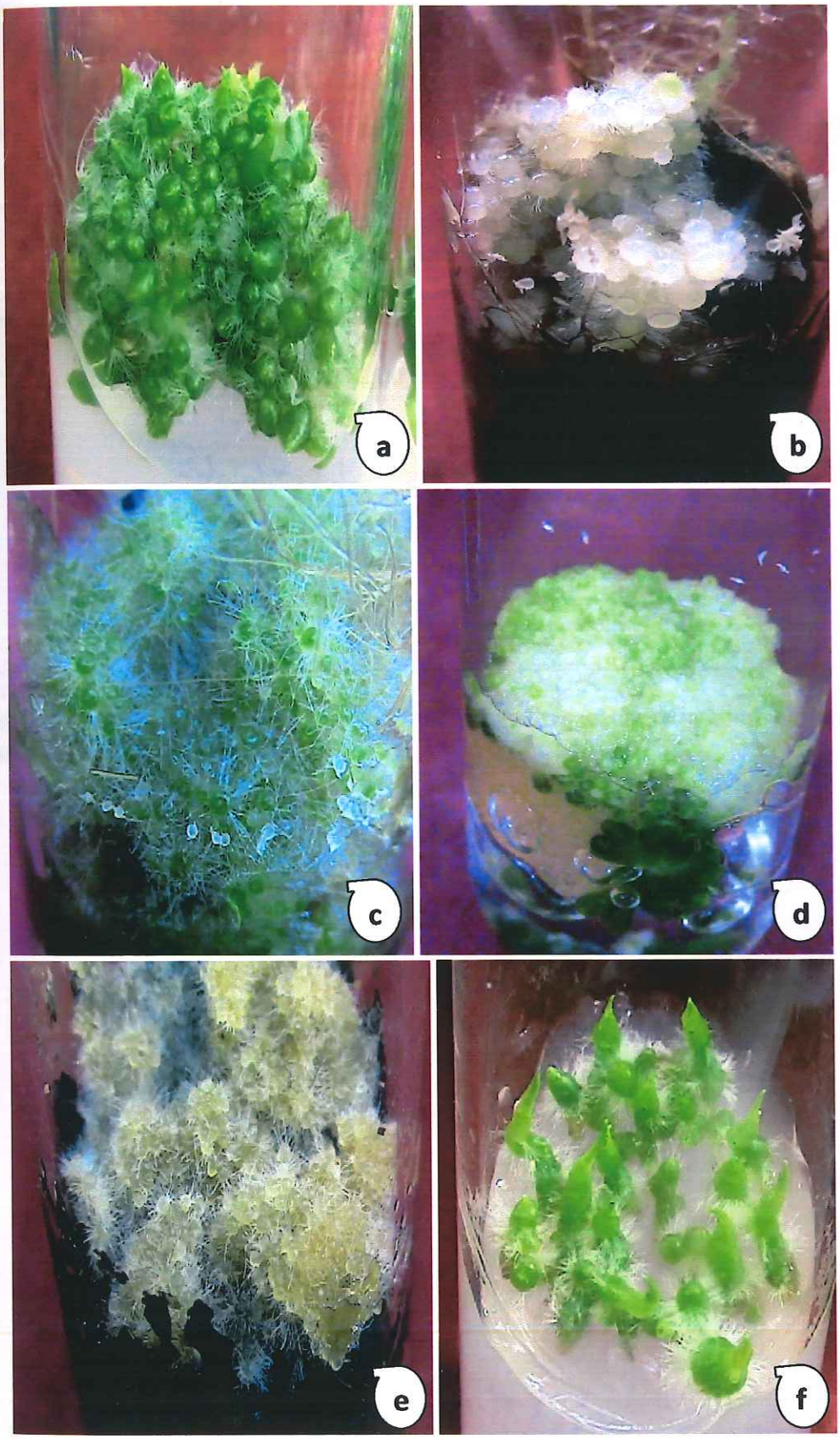

Figure - 3

Table 3: Effects of different basal media on non-symbiotic seed germination of *C. aloifolium*

Basal media	Germination time (days)	Germination rate (%) (±SE)*	Type of response**
Gamborg (B_5)	140	50 (2.00)	Nodular swelling
Knudson 'C'	150	50 (2.00)	Nodular swelling of the embryos with the formation of few PLBs but degenerated subsequently.
Mitra *et al*	130	70 (2.5)	Small green PLBs.
MS	58	90 (1.5)	Healthy green PLBs.
SH	160	30 (2.00)	Slight swelling only

* Standard error

** Media containing sucrose (2%) (w/v), NAA+BA (3+6 µM respectively in combination). Data represents the mean of three replicates.

Table 4: Effect of different basal media on non-symbiotic seed germination of *Cymbidium iridioides*

Basal medium	Germination time (days)	% Germination (±SE)*	Type of response**
Gamborg (B_5)	62	50 (1.25)	Few PLBs but no plantlets
Knudson 'C'	65	45 (1.00)	As above
Mitra *et al*	70	65 (1.50)	Moderate germination and healthy green PLBs formed but regeneration delayed
MS	58	95 (1.5)	Most of the germinated embryos converted into healthy PLBs
SH	75	50 (0.75)	Germinated seeds formed PLBs but failed to differentiate and degenerated

* Standard error; ** Media containing sucrose (2%, w/v), NAA+BA (3+3µM respectively in combination)

Data represents the mean of three replicates.

media namely B_5 and SH were significantly poorer as these two media did not support germination. Embryos cultured on MS medium supported into green and healthy protocorm like bodies (PLBs) formation while, cultures maintained on Mitra *et al* medium resulted smaller PLBs. A similar trend was also recorded with *C. iridioides* where within 58 days of culture as much as 95% seeds germinated and converted into healthy PLBs on MS medium followed by Mitra *et al* medium (65%) **(Table 4)**. The cultured seeds on MS medum supported formation of healthy PLBs, whereas Mitra *et al* medium supported moderate green PLBs formation. But in Knudson 'C' medium, only few green PLBs were formed followed by delayed differentiation.

Effects of Embryo/Seed Developmental Stage on *In Vitro* Culture Initiation

Green pod/capsule from both the selected species representing different physiological age was used for initiation of non-symbiotic seed germination. In both the species the developmental stages of the immature embryos showed a profound effect on successful non-symbiotic seed germination. Seed pod age of 9 MAP was found to be optimum for *C. aloifolium* where ~90% germination was registered after 58 days of culture while in the case of *C. iridioides,* seed pod age of 10 MAP recorded ~95% germination after 58 days of culture **(Table 1 & 2)**. In both species the young embryos either failed to germinate or required longer duration for germination. In case of *C. aloifolium* seed pod age up to 8 MAP showed delayed germination and did not support healthy seed germination. Seeds from green pod age up to 6 MAP from *C. iridioides* failed to germinate. With increase in green pod age, performance of seed germination improved substantially. The seeds from the older pods exhibited poor response accompanied by fungal contamination.

Table 5: Effect of different organic carbon sources on *in vitro* embryo culture of *C. aloifolium**

Organic carbon Sources & Conc. (%)	Days taken to germinate	% Germination (±SE)**	Type of response
0	80	60 (1.50)	Nodular swelling of the embryos.
Dextrose			
1	60	55 (1.00)	Few green PLBs.
2	55	60 (1.00)	As above.
3	55	65 (1.50)	Moderate germination
4	60	50 (1.00)	As above
Glucose			
1	70	50 (1.50)	Very few PLBs formed.
2	65	52 (2.00)	As above.
3	60	55 (2.50)	Very few healthy PLBs formed.
4	75	60 (2.00)	As above.
Sucrose			
1	65	70 (2.00)	Few green PLBs
2	58	90 (1.50)	Healthy green PLBs
3	54	75 (2.50)	PLBs formed.
4	90	60 (1.50)	Delayed germination.

* On MS medium containing NAA and BA (3 μM + 6 μM respectively) in combination and embryos scoped out from green pod of 9 MAP.

** Standard error

Data represents the mean of three replicates.

Table 6: Effect of different organic carbon sources on *in vitro* embryo culture on *C. iridioides**

Organic carbon sources	Conc. (%)	Days taken to germinate	% germination (±SE)**	Type of response
	0	98	50 (1.0)	Few PLBs formed
Dextrose	1	71	50 (1.5)	Very few PLBs formed
	2	70	70 1.5)	As above
	3	65	50 (1.0)	Very few healthy PLBs formed
	4	60	40 (1.0)	As above
Glucose	1	62	60 (1.5)	Few green PLBs formed
	2	60	65 (1.0)	As above
	3	55	75 (1.5)	Moderate germination but few green PLBs formed
	4	60	40 (1.0)	As above
Sucrose	1	62	75 (1.0)	Moderate germination but few green PLBs formed
	2	*58*	*95 (1.5)*	*Most of the germinated embryos converted into healthy PLBs with white hairy structures*
	3	65	85 (1.5)	As above but cultures were healthier in the above treatment
	4	95	65 (2.0)	Germination delayed and few healthy PLBs formed

* On MS medium containing NAA + BA (3 + 3 µM respectively in combination) and embryos scoped out from green pod of 10 MAP.

Data represents the mean of three replicates.

Table 7: Effect of coconut water (CW) on non-symbiotic seed germination of *C. aloifolium

CW Conc. (%, v/v)	Days taken to germinate	Germination % (±SE)**	Types of response
0	*58*	*90 (±1.50)*	*Healthy green PLBs formed*
5	56	80 (±0.75)	Healthy green PLBs formed
10	58	85 (±2.5)	Green PLBs formed.
15	55	70 (±2.5)	As above
20	54	60 (±2.0)	Few green PLBs formed.

* On MS medium containing sucrose (2%), NAA and BA (3 +6 µM respectively) in combination and green pod age of 9 MAP.
** Standard error.
Data represents the mean of three replicates.

Table 8: Effect of coconut water (CW) on non-symbiotic seed germination of *C. iridioides

CW Conc. (%, v/v)	Days taken to germinate	% germination (±SE)**	Types of response
0	*58*	*95 (±1.5)*	*Healthy green PLBs formed*
5	40	90 (±0.75)	Healthy green PLBs formed
10	40	75 (±2.5)	Green PLBs formed.
15	50	70 (±2.5)	As above
20	55	60 (±2.0)	As above

* On MS medium containing sucrose (2%), NAA and BA (3 + 3 μM respectively) in combination and green pod age of 10 MAP.

** Standard error.

Data represents the mean of three replicates.

Table 9: Effect of casein hydrolysate on non-symbiotic seed germination of *C. aloifolium

CH Conc. (mgl^{-1})	Days taken to germinate	Germination rate (%) (±SE)**	Types of response
0	*58*	*90 (±1.50)*	*Healthy green PLBs formed.*
50	60	70 (±1.25)	Green PLB s formed.
100	**55**	**80 (±2.00)**	**Healthy green PLBs formed.**
150	55	60 (±1.50)	Few green PLBs formed.
200	45	50 (±1.50)	As above

* On MS medium containing sucrose (2%), NAA + BA (3+6 µM respectively) in combination and green pod age of 9 MAP.

** Standard error

Data represents the mean of three replicates.

Table 10: Effect of casein hydrolysate (CH) on non-symbiotic seed germination of *C. iridioides

CH Conc. (mgl^{-1})	Days taken to germinate	Germination rate (%) (±SE)**	Types of response
0	*58*	*95 (±1.5)*	*Healthy green PLBs formed.*
50	48	70 (±2.00)	Green PLB s formed.
100	**48**	**85 (±0.75)**	**Healthy green PLBs formed.**
150	65	60 (±1.00)	Fewer PLBs formed
200	68	50 (±1.50)	As above

* On MS medium containing sucrose (2%), NAA and BA (3 + 3 μM respectively) in combination and green pod age of 10 MAP.

** Standard error.

Data represents the mean of three replicates.

Table 11: Effects of different levels of PGRS on non-symbiotic seed germination of *Cymbidium aloifolium*

PGRs Conc. (µM) NAA	BA	Germination time (Days)	Germination % (±SE)*	Type of response**
0	0	80	60 (1.50)	Nodular swelling of the embryos
3	0	77	80 (2.00)	Small green PLBs formed
6	0	90	50 (1.25)	Delayed formation of PLBs
9	0	90	45 (1.50)	Nodular swelling of embryos
0	3	45	70 (1.25)	As above
0	6	45	75 (0.75)	As above
0	9	77	70 (1.50)	As above
3	3	45	70 (1.00)	Healthy green PLBs formation
3	***6***	***58***	***90*** (1.50)	***Healthy green PLBs which were subsequently converted into plantlets***
3	9	90	60 (1.25)	Green PLBs formed
6	3	105	40 (2.00)	Delayed germination
6	6	45	50 (2.00)	Few PLBs formed
6	9	90	45 (1.50)	Nodular swelling of embryos
9	3	90	45 (1.50)	As above
9	6	85	50 (1.25)	PLBs formed subsequently degenerated
9	9	90	40 (1.25)	Delayed germination.

* Standard error

** On MS medium containing sucrose (2%) (w/v) and seed pod age of 9 MAP.

Data represents the mean of three replicates.

Effects of Different Organic Carbon Sources and Other Adjuncts on Asymbiotic Embryo Culture

Different concentrations of various organic carbon sources viz., dextrose, glucose and sucrose (0-4%, w/v), casein hydrolysate (CH) (0-200 mgl^{-1}) and coconut water (CW) (0-20%, v/v) were also incorporated in the germination media. Incorporation of one of the organic carbon in the initiation media was perquisite for successful germination of seeds/embryos. In *C. aloifolium* only nodular swelling of embryos was observed on media devoid of any organic carbon while, in case of *C. iridioides* germination was delayed and fewer PLBs formed. Amongst the different quality and quantity of organic carbons used, sucrose at a concentration of 2% supported optimum germination in both the species (**Table 5 & 6**). In both the species at lower concentration of sucrose, fewer PLBs were formed while at higher concentration, there was delayed germination. Both dextrose and glucose could not support healthy germination. In the preliminary studies, CW and CH were incorporated in the germination media. But no significant effects were recorded in the entire range of CW and CH studied. It was observed that in both CW and CH enriched initiation media the optimum responses were far behind the control in both the species under study (**Table 7-10**).

Effects of Quality and Quantity of PGRs on Asymbiotic Embryo Culture

Amongst the different levels of PGRs used for non-symbiotic seed germination of *C. aloifolium*, the MS medium containing NAA + BA (3.0 + 6.0 µM respectively) in combination supported optimum germination (~90%) after 58 days of culture and formation of healthy PLBs. The lone treatment of NAA delayed germination and formed fewer PLBs while, BA enriched media did not support healthy germination and only nodular swelling of embryos was observed. Only the combined treatment of NAA and BA supported healthy culture growth (**Table 11**). But MS medium containing NAA +

Table 12: Effect of plant growth regulators on *in vitro* culture of immature embryos of *C. iridioides*

PGRs Conc. (µM)[#]		Germination time (days)	% germination (±SE)[**]	Type of response[*]
NAA	BA			
0	0	63	40 (±1.5)	Small green PLBs formed
3	-	68	80 (±2.5)	Healthy green PLBs formation
6	-	75	60 (±2.0)	Green PLBs formed
9	-	85	60 (±1.5)	Delayed germination and few PLBs formed
12	-	85	50 (±1.5)	Small green PLBs formed and degenerated subsequently
-	3	67	50 (±2.0)	Few PLBs formed
-	6	79	50 (±2.5)	As above
-	9	98	30 (±1.00)	As above
-	12	98	30 (±1.50)	As above
3	***3***	***58***	***95 (±1.5)***	***The germinated embryos converted into green PLBs followed by release of first leaflets***
3	6	64	70 (±2.0)	Green PLBs formed
3	9	70	60 (±2.5)	As above but delayed germination
3	12	75	50 (±1.5)	Few PLBs formed but degenerated subsequently
6	3	103	50 (±2.0)	Delayed germination
6	6	64	65 (±1.5)	Fewer Green PLBs formed
6	9	105	55 (±2.5)	As above
6	12	98	40 (±1.50)	Nodular swelling, but no PLBs formation
9	3	64	45 (±2.0)	Few green PLBs formed
9	6	75	40 (±2.0)	As above but degenerated
9	9	98	30 (±1.0)	As above
9	12	98	30 (±1.00)	As above
12	3	105	35 (±1.5)	As above
12	6	110	40 (±0.5)	Delayed germination
12	9	105	35 (±1.00)	As above
12	12	105	33 (±1.00)	As above

* On MS medium containing sucrose (2%, w/v) and embryos of 10 MAP.
** Standard error
Data represents the mean of three replicates

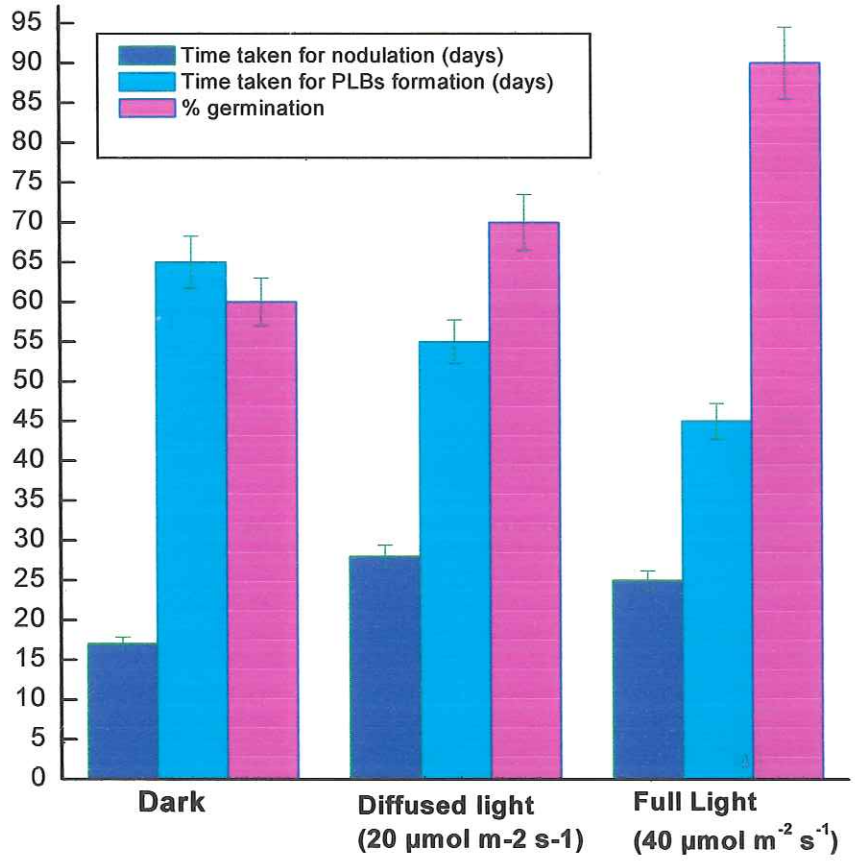

Figure –4

Figure 4: Effects of light quantity on *in vitro* culture of immature embryos of *Cymbidium aloifolium*

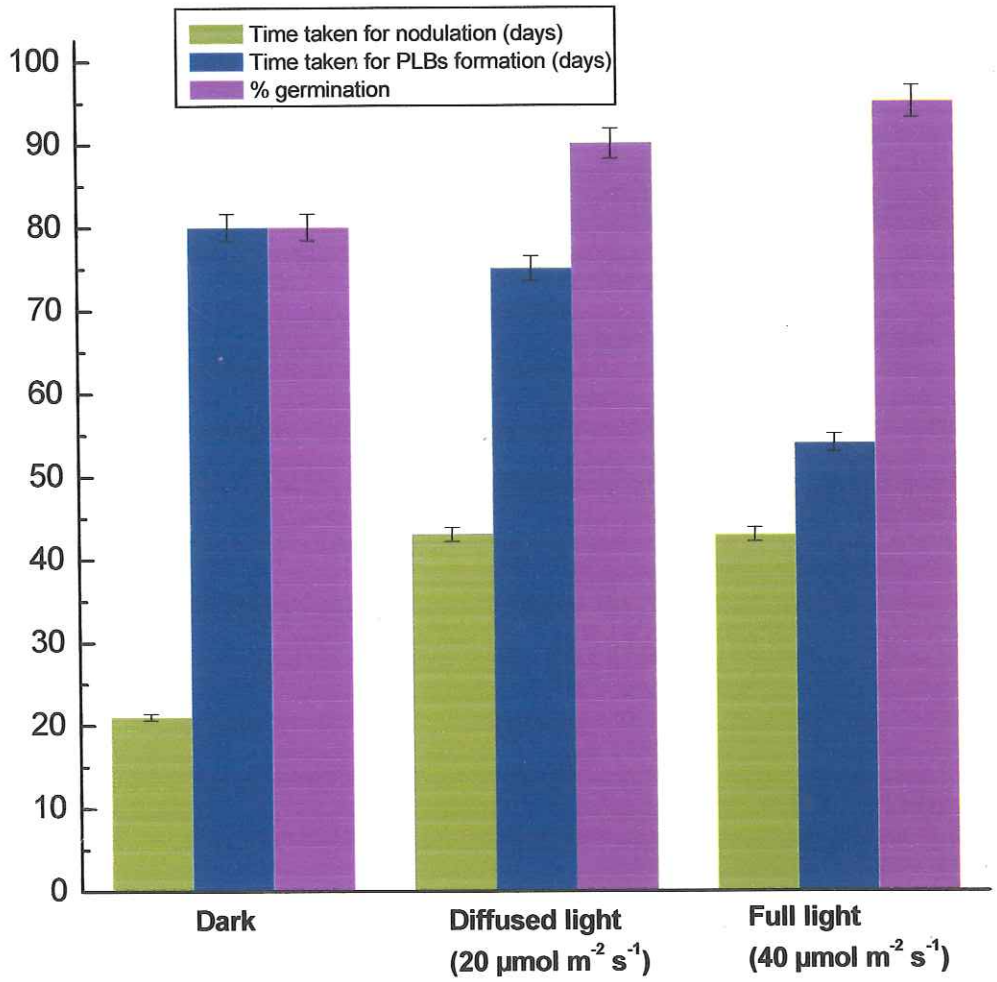

Figure –5

Figure 5: Effects of light quantity on *in vitro* culture of immature embryos of *Cymbidium iridioides*.

Table 13: Asymbiotic seed germination[@] of *C. aloifolium* on different alternative substratum*

Substratum	Days taken to germinate	Germination (%) (±SE)**	Types of response
Agar	58	80 (1.50)	All the embryos formed healthy and green PLBs.
Betel nut coir	49	65 (0.75)	Green PLBs formed but delayed differentiation.
Coconut coir	43	75 (1.00)	Green PLBs formed and delayed differentiation
Foam	45	90 (0.75)	Nodular swelling and green PLBs formation
Leaf litter	45	50 (1.50)	Nodular swelling but few green PLBs formed.

@ Seeds from green pod of 9 MAP; * On MS medium containing NAA and BA (3+6 µM respectively) in combination and sucrose (2%, w/v); ** Standard error.

Note: Data represents the mean of three replicates.

BA (3.0 + 3.0 µM respectively) in combination registered optimum germination for *C. iridioides* (95%). Media enriched with either NAA or BA singly supported fewer PLBs formation while combined treatments at higher concentrations either delayed germination or impaired germination **(Table 12)**.

Effects of Light on *In Vitro* Culture of Immature Embryos

In the present study, three different light conditions were tested for *in vitro* seed germination for both the species. In *C. aloifolium* nodular swelling of embryos was observed within 17 days in the dark and in *C. iridioides* a similar response was registered after 21days in the dark. **(Fig. 4 & 5)**. Though nodular swelling of seeds was faster in the dark, optimum germination of seeds was registered with cultures maintained under full light conditions (40 µ mol $m^{-2} s^{-1}$) in both the species and cultures maintained in the dark delayed germination. Cultures maintained in diffused light supported moderate germination.

Effects of Different Alternative Substratums on *In Vitro* Culture of Immature Embryos

Apart from agar other materials like betel-nut coir, coconut coir, foam disk and leaf litter could be successfully used with differential success for seed germination. In *C. aloifolium* germination rate was slightly higher (~90%) on media containing foam as substratum compared to agar gelled medium (80%) **(Table 13)**. Apart from higher germination rate, foam containing medium supported faster germination where within 45 days of culture seeds germinated and formed PLBs. Seeds germinated successfully and formed PLBs on agar gelled medium and media containing betel nut coir, coconut coir, foam and forest leaf litter as substratum within 58, 49, 43, 45 and 45 days respectively **(Table 13 & Fig. 2 a-e)**. But in case of *C. iridioides* agar gelled medium supported better germination (~95%) compared to media containing foam (85%), betel-nut coir (90%),

Table 14: A-symbiotic seed germination@ on *C. iridioides* on different alternative substratum*

Substratum	Days taken to germinate	Germination (%) (±SE)**	Types of response
Agar	58	95 (1.50)	All the embryos formed healthy and green PLBs
Foam	45	85 (0.75)	Nodular swelling and green PLBs formation
Betel nut coir	49	90 (0.75)	Green PLBs formed but delayed differentiation
Coconut coir	43	70 (1.00)	Green PLBs formed and delayed differentiation
Leaf litter	45	50 (1.50)	Nodular swelling but few green PLBs formed

@ Seeds from green pod of 10 months after pollination (MAP).

* On MS medium containing NAA + BA (3 +3 µM respectively in combination) and Sucrose (2%).

** Standard error.

Note: Data represent the mean of three replicates.

Figure 6: Different stages of *in vitro* morphogenetic response of foliar explants of *C. aloifolium* and *C. iridioides*. **a-b:** ***C. aloifolium***. a. Cultured leaf explant of *C. aloifolium* swelling at the basal part and initiation of shoot bud formation, **b.** PLBs/shoot buds formed from the cultured leaf; **c-d:** ***C. iridioides.*** **c.** Cultured leaf explant of *C. iridioides* shows initiation of swelling at the basal part of leaf explants, **d.** PLBs/shoot buds formed from the cultured leaf.

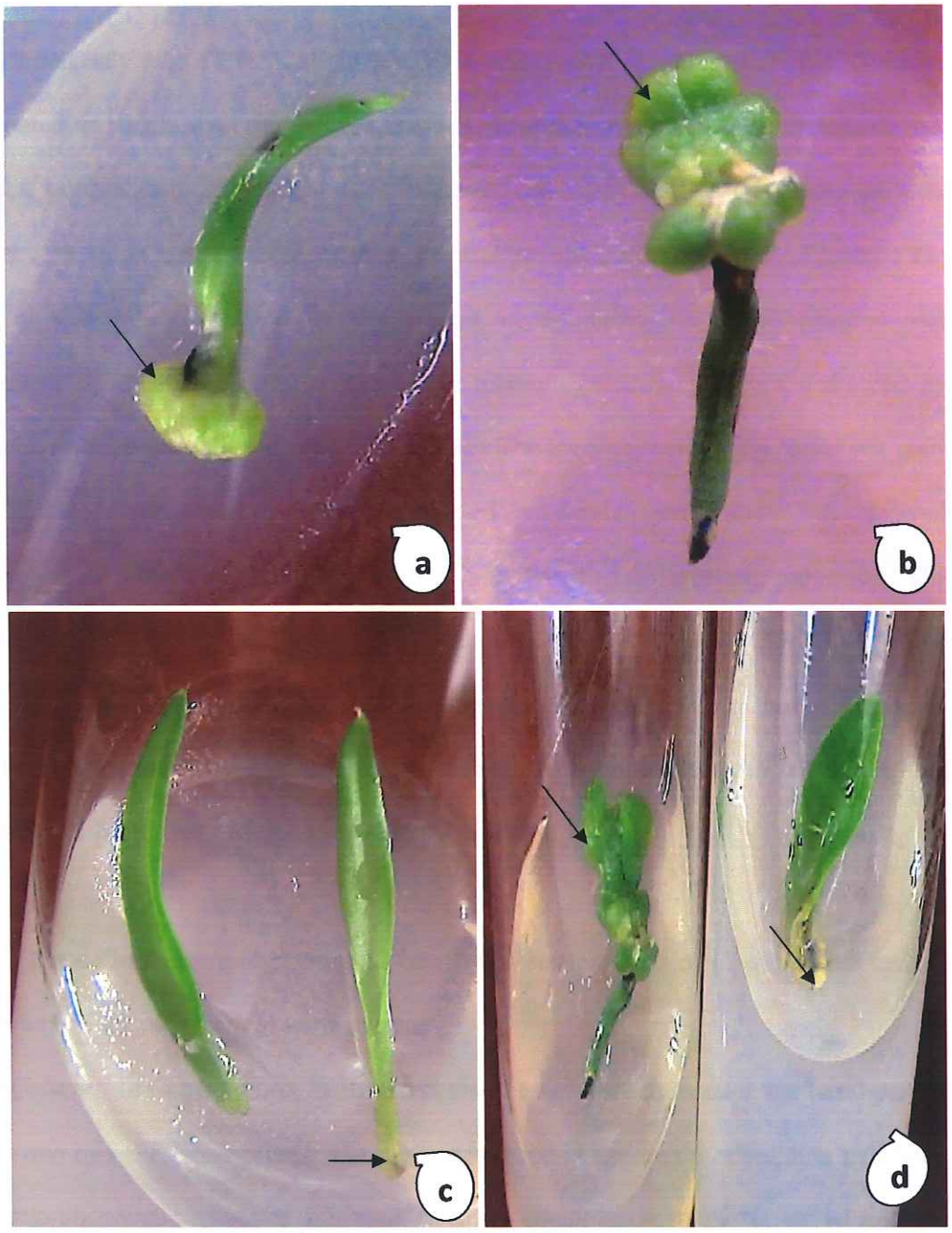

Figure - 6

coconut coir (70%) and leaf litter (50%) as substratums (**Table 14**). Though the germination rate was higher on agar gelled medium, it required longer duration compared to other substratums. The medium containing agar as gelling agent supported germination only after 58 days of culture while within 43 to 49 days of culture, embryos germinated on media with other substratum. Besides the time taken for germination and per cent germination, subsequent differentiation was equivalent to that achieved with agar and in some cases the alternative substratum out performed agar. Amongst the different substratums studied in the present investigation, better germination and subsequent differentiation was registered on medium containing 'Foam' followed by 'Coconut coir, 'Betel-nut coir' and 'Leaf litter'. The medium containing 'betel nut coir' and 'coconut coir' as substratum supported moderate germination but delayed differentiation, and cultures on chopped leaf litter failed to differentiate properly. The germinated seeds during subsequent sub-culture converted into PLBs (**Fig. 3 a-e**). The PLBs so formed on initiation media from immature embryos/seeds of both the species were maintained on the optimum initiation medium for 2 more passages and during this period the PLBs further differentiated and proliferated.

Leaf

The cultures were also initiated from the foliar explants of *C. aloifolium* and *C. iridioides* (~5 wk old) from *in vitro* source.

C. aloifolium: In *C. aloifolium* PLBs/direct shoot buds were induced in the basal part of the *in vitro* raised foliar explants. The explants placed in upside up orientation exhibited better morphogenetic response. Initiation of meristematic activity was observed initially at the basal ends of the explants after 7-8 wk of culture. Mostly the meristematic activity was restricted towards the lower basal parts (**Fig. 6a**). Amongst the various plant growth regulators (PGRs) tested for the initiation of culture, the single treatment of NAA and

Table 15: Effect of PGRs on morphogenetic response of foliar explants (~5 wk old) of *C. aloifolium* from *in vitro* source

PGRs Conc. (µM) NAA	BA	No. of regenerates/ meristemoids/explants	% response (±SE)*	Types of response**
Control		-	-	No response
3	-	-	-	Slight swelling at the base of the leaf
6	-	-	-	As above
9	-	-	-	Browning of leaves
-	3	-	-	Slight swelling at the base of the leaf
-	6	-	-	As above
-	9	-	-	No response and degenerated
3	3	-	-	Slight swelling at the base
3	6	2	25 (1.50)	PLBs formed from the basal part of leaf
3	9	3	20 (1.00)	As above
6	3	2	15 (0.75)	Slight swelling & PLBs formation
6	6	4	35 (0.75)	As above
6	***9***	***10***	***52 (1.50)***	***Multiple shoot buds/PLBs formed and differentiated into rooted plantlets***
9	3	2	17 (1.00)	Fewer shoot buds formed
9	6	-	-	No response
9	9	-	-	As above

* Standard error

** On MS medium containing sucrose (3%) (w/v)

Data represents the mean of three replicates.

Table 16: Effects of PGRs on morphogenetic response of foliar explants (~5 wk old) of *C. iridioides* from *in vitro* source

PGRs Conc. (µM) NAA	BA	No. of regenerates per explants	Time for response (days)	% response (±SE)*	Type of response**
Control		-	-	-	No response
3	-	-	50	20 (1.0)	Slight swelling at the base of the leaf
6	-	-	-	-	Browning of explants
9	-	-	-	-	No response
-	3	-	50	20 (1.5)	Slight swelling at the basal part of the leaf
-	6	-	-	-	Browning of leaves
-	9	-	-	-	As above
3	3	-	70	20 (1.5)	Slight swelling at the base of the leaf
3	6	4	30	60 (0.5)	PLBs formation at the basal part of the leaf
3	9	-	-	-	No response
6	3	8	90	90 (1.5)	Healthy PLBs formed
6	6	-	90	20 (1.0)	Slight swelling at the base of the leaf
6	9	-	-	-	No response
9	**3**	**20**	**90**	**60 (1.0)**	**Multiple PLBs formation**
9	6	-	90	20 (1.0)	Slight swelling at the base of the leaf
9	9	1	90	30 (1.5)	PLB formation

* Standard error

** On MS medium containing sucrose (3%) (w/v)

Data represents the mean of three replicates.

BA did not support morphogenetic response *in vitro*. Morphogenetic response was achieved only on MS medium containing both NAA and BA in combination. Lower concentrations of combined treatments exhibited poor morphogenetic response. About 52% cultures responded positively after 65 days of culture initiation on MS medium containing sucrose (3%) and NAA + BA (6 and 9 µM respectively in combination) where as many as 10 meristemoids invoked and formed shoot buds/PLBs (**Table 15 & Fig. 6b**). While at higher concentrations there was no response and cultures degenerated.

C. iridioides: Incorporation of PGRs in the initiation medium was absolute necessity. The cultured foliar explants remained recalcitrant on initiation medium containing no PGRs. The initiation medium containing either of the PGRs (NAA and BA) singly could not support morphogenetic response. At lower concentrations single treatment of both the PGRs supported only swelling of explants at the basal part. Morphogenetic response was initiated on medium containing NAA and BA in combination. Initiation of morphogenetic response was restricted to the basal part of the leaf explants (**Fig. 6c**). Under optimum condition, as many as 20 PLBs/shoot buds formed after 90 days of culture initiation. About 60% explants responded positively on MS medium containing sucrose (3%) and NAA + BA (9 + 3 µM respectively) in combination (**Table 16 & Fig. 6d**). The orientation of explants had little or no effect on morphogenetic response.

Aerial Root

For both the selected species besides immature seeds/embryos and foliar explants, cultures were also initiated from the aerial roots of *in vitro* source. The aerial root explants were collected from 5-6 wk old after emergence.

***Cymbidium aloifolium*:** In *C. aloifolium* the aerial root explants cultured on MS medium devoid of any PGRs remained recalcitrant. Initially some swelling was observed in few explants but degenerated subsequently. However, healthy PLBs and shoot buds were

Table 17: Effects of PGRs on culture initiation from aerial roots (5-6 wk old) of *C. aloifolium* from *in vitro* source

PGRs Conc. (µM) NAA	IAA	BA	KN	% response (±SE)*	Type of response)**
Control				10 (0.50)	Only swelling of roots
3	-	-	-	20 (0.50)	No distinct shoot bud formed, hair-like structure formed
6	-	-	-	-	No response
9	-	-	-	-	As above
-	3	-	-	35 (1.50)	1-2 shoot buds formed at the tip of the root
-	6	-	-	15 (0.50)	Only hairy structure observed
-	9	-	-	15 (0.50)	Only elongation of root tip is observed.
-	-	3	-	17 (1.00)	Only elongation of root tip is observed
-	-	6	-	20 (0.50)	Protrusions with hairy structures observed
-	-	**9**	-	**35 (1.25)**	**Multiple PLBs formed**
-	-	-	3	60 (1.00)	Few shoot buds formed on both ends of the root and subsequently differentiated into healthy plantlets
-	-	-	6	20 (1.00)	Only elongation of root
-	-	-	9	10 (0.50)	As above
3	-	3	-	15 (0.50)	As above
3	-	6	-	10 (0.50)	As above
3	-	9	-	14 (1.00)	As above
6	-	3	-	17 (1.00)	As above
6	-	6	-	15 (0.50)	As above
6	-	9	-	10 (0.50)	As above
-	3	-	3	20 (1.00)	As above
-	3	-	6	10 (0.50)	Only hairy structures observed.
-	3	-	9	10 (0.50)	Hairy structures observed
-	6	-	3	25 (1.50)	Hairy structure observed
-	**6**	-	**6**	**38 (0.50)**	**Multiple PLBs formed**
-	6	-	9	25 (1.50)	Hairy structures observed
-	9	-	3	20 (1.00)	As above
-	**9**	-	**6**	**45 (1.00)**	**Shoot buds formed at the tip of the roots**
-	9	-	9	20 (1.00)	Hairy structure observed

* Standard error; ** On MS medium containing sucrose (3%) (w/v).
Data represents the mean of three replicates.

Figure 7: Stages involved in *in vitro* culture of aerial roots of *C. aloifolium* and *C. iridioides*. **a-b:** ***C. aloifolium*: a.** Initiation of culture from aerial root segments showing swelling and hairy PLB formation, **b.** Multiple shoot buds / PLBs formed from the cultured roots. **c-d:** ***C. iridioides*: c.** Initiation of culture from aerial root segments showing swelling and PLBs formation, b. Shoot buds and plantlets formed from the responding root segment.

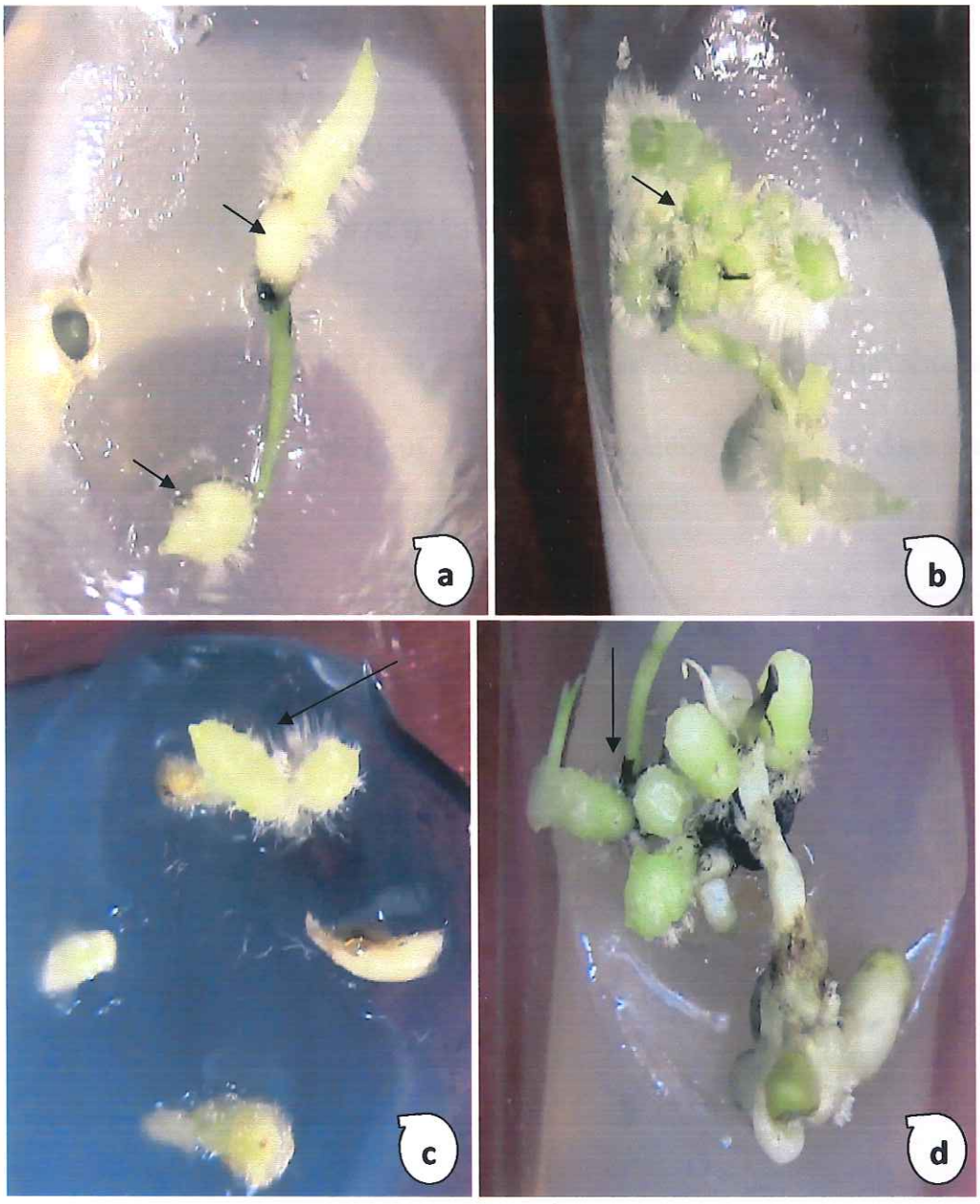

Figure - 7

Table 18: Effects of activated charcoal (AC) on *in vitro* morphogenetic response of aerial roots of *C. iridioides* from *in vitro* source (~5-6 wk old)

AC Conc. (%) (w/v)	Days taken for meristemoid formation	% response (±SE)*	Type of response**
0	0	-	No response
0.05	50	30 (1.0)	Only elongation of root tips
0.10	***40***	***50 (0.5)***	***Swelling of root tips followed by PLBs formation***
0.15	40	40 (1.5)	Slight swelling but no PLBs formation
0.20	40	40 (1.0)	Slight swelling of roots but degenerated subsequently

* Standard error
** On MS medium containing sucrose (3%, w/v) and IAA (3 µM).
Data represents the mean of three replicates.

Table 19 : Effect of plant growth regulators on culture initiation of *C. iridioides* aerial roots (5-6 week old) from *in vitro* source.

PGRs conc. (µM) NAA	IAA	BA	KN	% Response (±SE)*	Type of response**
0	0	0	0	-	No response
3	0	0	0	-	Hair-like structures observed.
6	0	0	0	-	No response
9	0	0	0	-	No response
0	**3**	**0**	**0**	**30 (1.00)**	**Swelling followed by PLBs formation at the tip of the root.**
0	6	0	0	20 (1.00)	Swelling of roots and hair-like structures observed but no PLBs formation.
0	9	0	0	10 (0.50)	As above.
0	0	3	0	15 (1.00)	Only elongation of the root tip is observed.
0	0	6	0	10 (1.00)	As above
0	0	9	0	10 (1.00)	As above
0	0	0	3	16 (1.50)	Slight swelling at the tip of the root.
0	0	0	6	20 (2.00)	Elongation with hairy structures followed by one PLB formation
0	0	0	9	10 (1.00)	Elongation with hairy structures
3	0	3	0	05 (1.00)	As above
3	0	6	0	05 (0.50)	As above
3	0	9	0	-	As above
6	0	3	0	-	As above
6	0	6	0	-	As above
9	0	3	0	-	As above
9	0	6	0	-	As above
9	0	9	0	-	As above
0	3	0	3	-	As above
0	3	0	6	-	As above
0	3	0	9	-	No response
0	6	0	3	-	As above
0	6	0	6	-	As above
0	6	0	9	-	As above
0	9	0	3	15 (1.00)	Hairy structures observed at the tip of the root.
0	9	0	6	15 (1.50)	As above
0	9	0	9	10 (1.00)	As above

* Standard error

** On MS medium containing sucrose (3%) (w/v) and AC (0.1%).

Data represents the mean of three replicates.

Table 20: Effects of PGRs on morphogenetic response of nodal explants of *C. aloifoium* from *in vitro* source

PGR Conc. (µM)		% response (±SE)*	Time for culture initiation (days)	No. of shoot/PLBs formed/Node	Types of response**
NAA	BA				
Control		60 (0.50)	5	1	Fewer shoot buds formed
3	-	70 (1.50)	13	2	Shoot bud differentiated into plantlets
6	-	80 (1.50)	13	5	As above
9	-	90 (1.00)	18	5	As above
-	3	50 (0.50)	15	1	One shoot bud invoked
-	6	88 (1.25)	18	3	PLBs differentiated into plantlets
-	9	90 (1.50)	14	4	As above
3	3	55 (2.00)	13	1	As above
3	6	60 (1.00)	18	3	PLBs formed at the node
3	9	58 (1.50)	14	2	As above
6	3	72 (1.00)	15	2	Direct shoot buds formed
6	6	48 (0.50)	21	2	As above
6	9	80 (1.00)	18	1	As above
9	3	60 (2.00)	18	2	As above
9	6	30 (1.00)	20	1	Shoot bud failed to differentiate
9	9	30 (1.00)	15	1	As above

* Standard error; ** On MS medium containing sucrose (3%).

Data represents the mean of three replicates.

Figure 8: Different stages of *in* vitro morphogenetic response of nodal explants. **(a-b: *C. aloifolium* and c-d: *C. iridioides*). a.** Sprouting of new shoot buds from the cultured nodes, **b**. Multiple shoots developed from the nodal segment; **c.** Sprouting of new shoot buds from the cultured nodal explants of *C. iridioides* and **d.** Multiple shoots/buds formed from the cultured nodal explants.

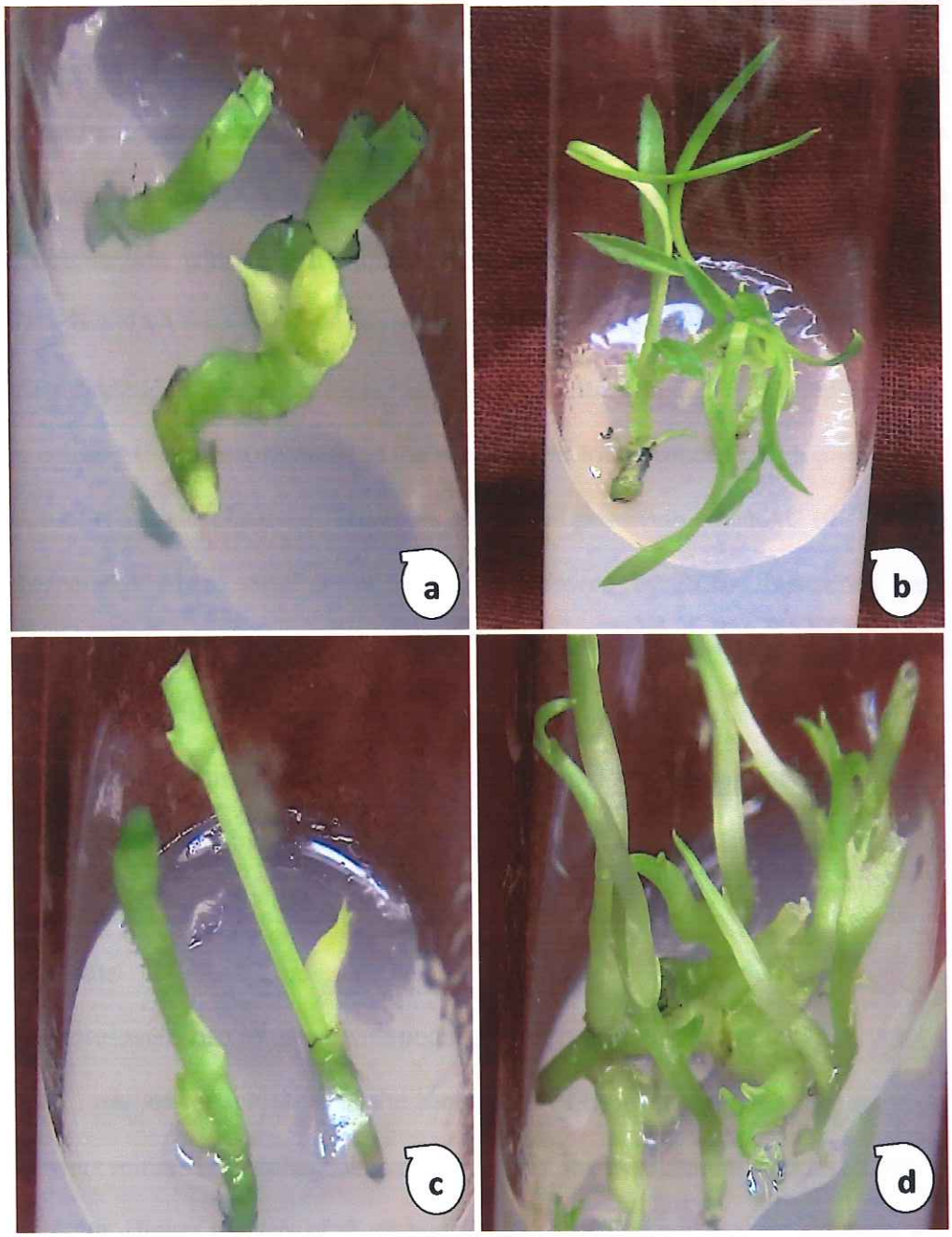

Figure - 8

formed from the culture of *in vitro* sourced aerial roots after 20 days of culture initiation. Of the four different PGRs tested, except kinetin (KN) other PGRs singly could not support healthy culture initiation. Amongst the single treatments, MS medium containing sucrose (3%) and KN (3 µM) supported the optimum morphogenetic response (60%) where shoot buds and PLBs formed at both ends of the root (**Table 17 & Fig. 7a**). Both the auxin sources used (IAA and NAA) singly impaired regeneration. When IAA was used in conjunction with KN, it improved the morphogenetic response significantly (**Table 17**), but NAA used in combination with BA did not improve the response.

***Cymbidium iridioides*:** The aerial roots remained recalcitrant on different PGRs enriched medium unless AC was incorporated in the medium. At lower concentration of AC, only elongation of roots was observed. The roots responded positively when AC (0.1%, w/v) was incorporated where swelling of root tips followed by PLBs formations were observed after 40 days of culture (**Table 18**). At higher concentration of AC, cultures degenerated without differentiation. Optimum morphogenetic response of aerial root was recorded on MS basal medium containing sucrose (3%), AC (0.1%) and IAA (3 µM) where as many as 50% cultures responded positively and formed PLBs (**Table 19 & Fig. 7c**).

Nodal explants

Cultures were also initiated with nodal segments of both the species from *in vitro* source. In *C. aloifolium* sprouting of the shoot buds/PLBs from the nodal region of the segments were initiated. Morphogenetic response was observed from the nodes within 15 days of culture. Higher number of shoot buds/PLBs formation and percent response was registered with single treatments of NAA and BA than with the combined treatments. As many as 5 shoot buds/PLBs developed at a single node on MS medium supplemented with sucrose (3%) and NAA (9 µM) after 18 days of culture (**Table 20 & Fig. 8a**).

Table 21: Effects of PGRs on morphogenetic response of nodal explants of *C. iridioides* from *in vitro* source

PGRs Conc. (µM) NAA	BA	Time for 1st meristemoid (days)/(No. of Shoot buds)	% response (±SE)*	Type of response**
0	0	20/1	60 (1.00)	Shoot bud formation.
3	0	4/ 6	60 (0.75)	Healthy shoot buds formation from single nodal region subsequently differentiated into plantlets.
6	0	10/ 2	60 (1.50)	Shoot buds formation on a nodal region.
9	0	40/2	40 (1.00)	As above
0	3	10/5	80 (1.50)	Shoot buds on a node with the formation of a secondary PLB.
0	6	15/2	60 (2.00)	Shoot buds on a nodal region.
0	9	40/2	20 (1.00)	As above
3	3	10/4	80 (1.50)	Shoot buds on each nodal region which further differentiates into plantlets with leaves and roots.
3	6	10/6	60 (0.75)	Shoot buds formed from single node which differentiates into plantlets with secondary PLBs.
3	9	15/1	80 (1.50)	One shoot bud on a node
6	3	14/3	80 (1.50)	Shoot buds on a node
6	6	14/3	40 (1.50)	As above
6	9	15/1	60 (1.00)	One shoot bud per node followed by plantlet formation.
9	3	40/1	40 (2.00)	As above
9	6	40/2	20 (1.50)	As above
9	9	40/1	20 (1.00)	As above

* Standard error

** On MS medium containing sucrose (3%),

Data represents the mean of three replicates.

Table 22: Effects of different basal media on regeneration of plantlets of *C. aloifolium

Basal media	No. of shoot/PLBs formed/explants	Time taken for 1st leaf (days)	Response*
Knudson 'C'	1	35	Elongation of shoot bud but degenerated subsequently
Mitra *et al*	3	16	Plantlets healthy but stunted in growth with fewer leaves and roots
MS	12	21	Healthy plantlets with 3-4 roots and multiple shoot buds and PLBs

* Media containing sucrose (3%) (w/v), BA (3 μM).

Data represents the mean of three replicates.

Table 23: Effect of different basal media on regeneration of plantlets of *Cymbidium iridioides*

Basal medium	No. of shootbuds formed / explant	Time taken for formation of 1st leaf (days)	Time taken for formation of 1st root (days)	Response*#
Knudson 'C'	1	20	-	Browning of the PLBs observed.
Mitra *et al*	4	18	80	Plantlets healthy, stunted with leaves and roots.
MS	20	16	20	Well-rooted healthy plantlets with multiple shoot buds.

* On media containing sucrose (3%) (w/v), CH (100 mgl^{-1}), CW (15%, v/v), NAA and BA (3 µM and 6µM respectively) in combination.
# On Agar gelled medium.
Data represents the mean of three replicates.

About 90% explants responded positively with the sprouting of shoot buds/PLBs from the nodal regions under optimum conditions.

From the nodal segments of *C. iridioides* optimum response was recorded on MS medium fortified with sucrose (3%) and NAA (3 µM) where as many as 6 shoot buds/PLBs were developed from a node after 10 days of culture (**Table 21 & Fig. 8c**). Positive response was recorded with both single and combined treatments of NAA and BA.

The PLBs and shoot buds formed from the cultured immature embryos/seeds, foliar explants, aerial roots, nodal segments of both the species were maintained for another 2 passages for further differentiation and proliferation on optimum growth conditions (**Fig. 2f, 3f, 6b, d, 7b, d, 8b, d**). The advanced stage PLBs, shoot buds developed from the germinated seeds, foliar explants, aerial roots and cultured nodal explants were subjected to regeneration of plantlets and mass multiplication.

Regeneration and Maintenance of Cultures

The advanced stage PLBs/shoot buds developed from the cultured immature embryos/seeds, leaf, root and nodal explants of *C. aloifolium* and *C. iridioides* were maintained further for 2 passages on the optimum initiation conditions for further differentiation.

Effect of Basal Media on Regeneration and Mass Multiplication

The differentiated PLBs, shoot buds and young plantlets were maintained on three different media viz., Knudson 'C', Mitra *et al* and MS media with different growth adjuncts. In the preliminary study it was observed that MS medium supported better regeneration and multiplication of culture in both the species (**Table 22, 23**) compared to the other two media. In *C. aloifolium* as many as 12 shoot buds/PLBs developed per explant per subculture where as in *C. iridioides* as many as 20 shoot buds developed

Figure 9: Plantlets regeneration and multiple shoots/buds formation of *C. aloifolium* on regeneration medium containing different substratums. **a.** Multiple shoots/buds/PLBs formed on agar gelled regeneration medium; **b.** Multiple shoots/buds/PLBs formed on regeneration medium containing foam as substratum; **c.** Multiple shoots/buds/PLBs formed on regeneration medium containing coconut coir as substratum.

Figure - 9

Figure 10: Plantlets regeneration and multiple shoots/buds formation of *C. iridioides* on regeneration medium containing different substratums. **a.** Multiple shoots/buds/PLBs formed on agar gelled regeneration medium; **b.** Multiple shoots/buds/PLBs formed on regeneration medium containing foam as substratum; **c.** Multiple shoots/buds/PLBs formed on regeneration medium containing coconut coir as substratum.

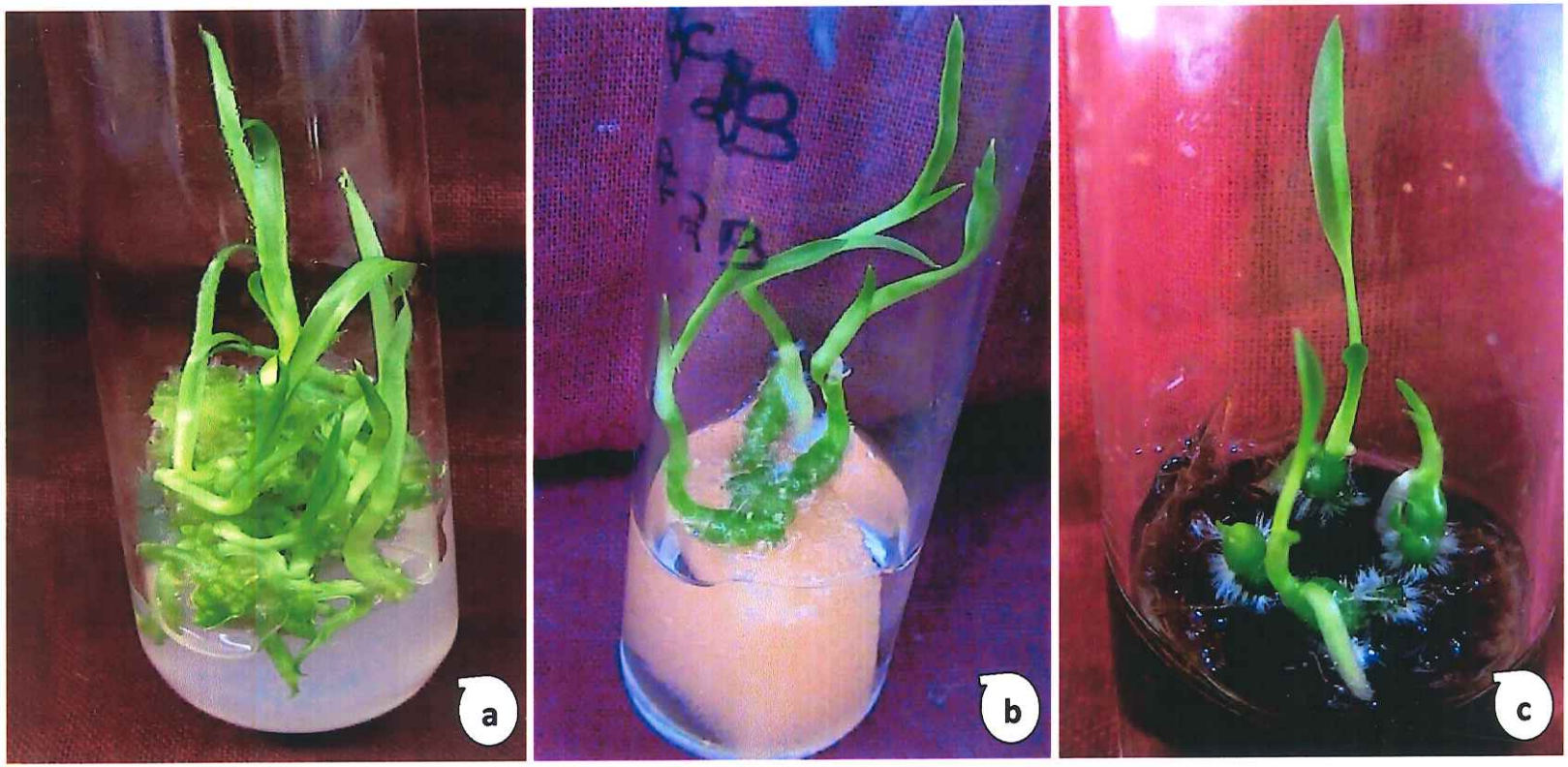

Figure - 10

Table 24: Effects of different strengths of MS medium for PLBs differentiation, plant regeneration and mass multiplication of *C. aloifolium*

Strength of MS medium	Avg. plant height (cm.)	No. of shoot buds formed/subculture	Type of response*
Control	-	-	Culture degenerated
1/4th	1.0	2	Plantlets stunted in growth with few small leaves
1/2	2.0	4	Plantlets etiolated but with small leaves
3/4th	3.5	5	Plantlets etiolated with well developed leaves and roots
Full	6.0	12	Well rooted healthy plantlets

* Media containing sucrose (3%) (w/v) and BA (3µM).

Data represents the mean of three replicates.

Table 25: Effect of different strengths of MS medium for PLBs differentiation, plantlet regeneration and mass multiplication of *C. iridioides*

Strength of MS medium	Avg. Plantlet height. (cm)	No. of shoot buds formed/subculture	No. of roots per plantlet	Type of Response*
0	-	-	-	-
1/4th	2.5	1	1	Plantlets stunted in growth with small leaves.
½	2.0	3	2	Plantlets stunted but with large number of leaves.
3/4th	3.0	5	2	Plantlets healthy.
Full	5.5	20	3	Well rooted healthy plantlets.

* On different strengths of MS medium containing sucrose (3%) (w/v), CH (100 mgl^{-1}), CW (15%, v/v), NAA and BA (3 µM and 6 µM respectively) in combination.

Data represents the mean of three replicates.

Table 26: Effects of different organic carbon sources on plant regeneration and mass multiplication of *C. aloifolium*

Organic carbon source & Conc. (%)	No. of shoot/PLBs formed/explants	Time taken for 1st leaf formation	Type of response*
Dextrose			
0	-	-	No regeneration
1	3	17	Plantlet growth stunted
2	4	17	Plantlets with fewer small leaves
3	6	17	Plantlets healthy with broader leaves with fewer roots
4	8	40	Well rooted healthy plants
Glucose			
0	-	-	Culture degenerated
1	2	17	Stunted growth and eventually degenerated
2	2	17	As above
3	2	34	Plant growth retarded
4	3	72	As above
Sucrose			
0	-	-	No regeneration
1	2	17	Plantlets stunted in growth
2	3	17	As above
3	12	21	Well rooted plantlets with multiple shoot buds/PLBs
4	4	17	Plantlets stunted and turned brown subsequently

* On MS medium containing BA (3µM)

Data represents the mean of three replicates.

(Fig. 9a & 10a). In both the species cultures differentiated faster on MS medium where sprouting of leaves was faster compared to other two media.

Subsequent experiments were conducted to study the effects of different strengths of MS medium on plant regeneration and plant growth **(Table 24 & 25)**. Amongst the different strengths of MS medium studied in the present investigation, in *C. aloifolium* it was found that at lower strengths ($1/4^{th}$, 1/2 strengths) plant growth was stunted accompanied by fewer new shoot bud formation. Optimum plant height (~6 cm) as well as maximal shoot buds formation (12 shoot buds per plant per subculture) were achieved on full MS medium **(Table 24)**. A similar response was also registered with *C. iridioides* where on full MS medium as many as 20 shoot buds formed with the average plant height of ~5.5 cm **(Table 25)**. At lower strengths, plants exhibited stunted growth along with fewer leaves and roots.

Effect of Different Organic Carbon Sources on Regeneration and Mass Multiplication

The basal media were fortified with different levels of various organic carbon sources like dextrose, glucose and sucrose (0-4%) besides other adjuncts. In *C. aloifolium,* of the three different organic carbon sources incorporated in the regeneration medium, cultures maintained on sucrose rich media outperformed the cultures maintained on media with other organic carbon sources under otherwise identical condition. The cultures maintained on glucose enriched medium did not support regeneration and degenerated subsequently while, dextrose nourished medium supported poorer regeneration with fewer secondary shoot buds formation. Optimum regeneration was achieved when MS medium was conjunct with sucrose (3%) **(Table 26)**. While, in *C. iridioides*, lower concentration of glucose (1%) was found to be superior over other two organic carbon sources, but at higher concentration, sucrose was registered as better

Table 27: Effect of different organic carbon sources on plantlet regeneration and mass multiplication of *C. iridioides*.

Organic carbon source	Conc. (%)	No. of shoot buds formed/explants	1st leaf formation (Days)	Type of Response*
Dextrose	0	-	-	Explants degenerated.
	1	4	50	Plantlet growth retarded.
	2	3	14	Plantlets stunted with few small leaves.
	3	2	17	Plantlets slightly etiolated.
	4	3	17	Plantlets healthy.
Glucose	0	-	-	Explants degenerated.
	1	6	50	Plantlets slightly etiolated with few small leaves.
	2	3	17	Plantlets with few leaves.
	3	2	40	Plantlets with few leaves.
	4	2	40	Plantlet slightly stunted in growth.
Sucrose	0	-	-	Delayed differentiation.
	1	6	40	Plantlets healthy though stunted.
	2	10	40	As above.
	3	**20**	**16**	**Well-rooted healthy plantlets with multiple shoot buds.**
	4	1	50	Plantlets with small leaves.

* On MS medium containing CH (100 mgl^{-1}), CW (15%, v/v), NAA and BA (3 µM and 6 µM respectively) in combination.
Data represents the mean of three replicates.

Table 28: Effect of casein hydrolysate and coconut water on plantlet regeneration and mass multiplication of *C. aloifolium

Conc. of CH (mgl^{-1})	CW (%)	Time for 1st shoot bud formation (Days)	No. of shoot buds per explant (Days)	Time taken for 1st leaf	Type of response
0	0	17	12	21	Healthy plantlets.
50	-	27	2	27	Stunted in growth.
100	-	21	10	18	Plantlets healthy.
150	-	27	4	27	Plantlets etiolated.
200	-	27	3	20	Plantlets healthy but growth stunted.
-	5	27	2	30	Plantlets stunted.
-	10	27	6	27	Plantlets etiolated.
-	15	21	10	20	Healthy plantlets.
-	20	27	4	27	Plantlets etiolated.
50	5	25	3	20	As above
50	10	20	3	20	As above
50	15	20	4	18	As above
50	20	22	5	20	Plantlets turned brown
100	5	25	7	20	As above
100	10	15	10	15	Green plantlets
100	15	14	10	22	Healthy plantlets
100	20	17	6	25	Healthy plantlets
150	5	15	7	16	Plantlets etiolated.
150	10	15	6	15	As above
150	15	14	4	18	Plantlets turned brown
150	20	17	4	18	As above
200	5	20	2	20	As above
200	10	20	1	20	As above
200	15	20	1	25	As above
200	20	-	-	-	No response and turned brown

* On MS medium containing sucrose (3%) and BA (3 μM).
Data represents the mean of three replicates

Table 29: Effect of casein hydrolysate and coconut water on plantlet regeneration and mass multiplication of *C. iridioides

Conc. of CH (mgl^{-1})	CW (%)	Time for 1st shoot buds formation (Days)	No. of shoot buds per explant (Days)	Time taken for 1st leaf	Type of response
0	0	17	12	21	Healthy plantlets.
50	-	27	6	22	Stunted in growth.
100	-	21	12	14	Plantlets healthy but stunted.
150	-	14	8	14	Plantlets etiolated.
200	-	18	8	20	Plantlets healthy but growth stunted.
-	5	35	5	42	Plantlets stunted.
-	10	15	8	14	Plantlets etiolated.
-	**15**	**14**	**20**	**16**	**Healthy plantlets but pale green.**
-	20	25	2	14	Plantlets etiolated.
50	5	25	3	20	As above
50	10	20	4	18	As above
50	15	20	4	18	As above
50	20	22	3	15	Plantlets turned brown
100	5	25	4	20	Plants stunted in growth
100	10	15	10	15	Green plantlets
100	***15***	***12***	***20***	***14***	***Healthy and dark green shoots***
100	20	12	9	18	Healthy plantlets but slightly brown
150	5	15	6	16	Plantlets etiolated.
150	10	15	7	15	As above
150	15	14	7	18	Plantlets turned brown
150	20	17	5	18	As above
200	5	20	3	20	As above
200	10	20	2	20	As above
200	15	20	2	25	As above
200	20	-	-	-	Culture degenerated

* On MS medium containing sucrose (3%) and NAA + BA (3 + 6 μM respectively) in combination. Data represents the mean of three replicates

option for faster culture differentiation and proliferation (**Table 27**). Optimum regeneration and culture multiplication was accomplished on MS medium containing sucrose (3%) where as many as 20 shoot buds formed per plant per subculture.

Effect of CH and CW on Regeneration and Mass Multiplication

Apart from other adjuncts, CH and CW were also incorporated at variable concentrations (CH: 0-200 mgl^{-1} and CW: 0-20%) in the regeneration medium. In general, incorporation of CH and CW singly in the regeneration media had little effect in *C. aloifolium* (**Table 28**). Single treatment of CH at a concentration of 100 mgl^{-1} and CW at a concentration of 15% was found to be superior over other concentrations studied. However, the performances with optimum concentrations of CH and CW were far below in comparison to control.

In *C. iridioides*, both CH and CW singly in the regeneration medium had no promotory effect on regeneration of plants and culture proliferation. Plants raised on medium enriched with CH alone supported healthy and green plants formation but were stunted in growth, while cultures on medium nourished with CW alone supported well rooted plant formation but were slightly pale green in colour. However, formation of healthy, green and well rooted plants were achieved on MS medium containing both CH (100 mgl^{-1}) and CW (15%) in combination (**Table 29**).

Effect of PGRs on Regeneration and Mass Multiplication

The differentiated PLBs and or the young plantlets obtained from germinated immature embryos, leaf, root, and nodal explants of both the species were subjected to regeneration and mass multiplication. The different plant growth regulators exhibited differential effect on regeneration and mass multiplication.

Cymbidium aloifolium: Amongst the different quality and quantity of PGRs used, a lone treatment of **BA** (3 µM) supported optimum regeneration and mass multiplication of

Table 30: Effects of different PGRs on plant regeneration and mass multiplication of *C. aloifolium

PGRs Conc. (µM)**				No. of shoot/PLBs formed/explants	Days taken for		Type of response
NAA	IAA	BA	KN		1st leaf	1st root formation	
Control				1	86	-	Plant growth stunted
3	-	-	-	4	13	38	Plant growth moderate with few roots
6	-	-	-	4	38	86	Plantlets healthy with few PLBs
9	-	-	-	2	38	86	Plantlets etiolated
-	3	-	-	1	38	-	Plantlet slender & etiolated
-	6	-	-	1	86	-	As above
-	9	-	-	1	86	-	As above
-	-	*3*	-	*12*	*21*	*21*	***Healthy plantlets with multiple shoot buds***
-	-	6	-	8	25	86	Plantlets healthy
-	-	9	-	7	25	86	As above
-	-	-	3	3	21	21	As above
-	-	-	6	3	10	51	Plantlets growth stunted
-	-	-	9	3	13	165	Poor rooting and degenerated subsequently
3	-	3	-	5	21	77	Growth stunted
3	-	6	-	4	21	-	As above
3	-	**9**	-	**10**	**38**	**86**	**Plant growth stunted**
6	-	**3**	-	**9**	**13**	**86**	**Healthy plant growth**
6	-	6	-	2	38	-	Few PLBs formed
6	-	9	-	2	38	-	As above
9	-	3	-	3	13	86	Plant growth stunted
9	-	6	-	8	38	86	As above
9	-	9	-	2	38	-	As above
-	3	-	3	2	21	-	Plantlets etiolated
-	3	-	6	1	38	-	As above
-	3	-	9	1	38	-	Plantlets stunted
-	6	-	3	1	38	-	As above
-	6	-	6	2	30	-	As above
-	6	-	9	1	38	-	As above
-	9	-	3	4	13	165	Plantlets etiolated, pale green
-	9	-	6	1	13	-	Slightly elongated
-	9	-	9	1	38	-	As above

* On MS medium containing sucrose (3%) (w/v).
** Only the significant treatments are computed here.
Data represents the mean of three replicates.

plantlets which produced multiple shoots/buds (**Fig. 9 a, b, c**). As many as 12 shoots/buds developed per explant per subculture and within 21 days of culture first leaf and root sprouted (**Table 30**). Of the four different PGRs tested, NAA and IAA singly did not support healthy regeneration and in most of the cases either plants were etiolated or fewer shoot buds were formed. While, both BA and KN singly supported better regeneration and culture proliferation and BA was found to be superior over KN.

Though the single treatments with either NAA or IAA did not support healthy culture differentiation, combined treatments of NAA + BA were found to be beneficial. A combined treatment of NAA + BA (6 + 3 μM respectively) exhibited faster culture differentiation compared to lone treatment of BA where first leaf was released after 13 days of culture initiation (**Table 30**). But combined treatments of IAA and KN were found to be inhibitory and failed to support regeneration as well as culture proliferation and in most of the cases cultures failed to produce roots. Other combinations like NAA + KN and IAA + BA did not support any regeneration and cultures degenerated subsequently.

***Cymbidium iridioides*:** The advanced stage PLBs from cultured seeds started converting into young rooted plantlets and repetitive PLBs within 3-4 wk on regeneration media. It was observed that the single treatment of different PGRs at lower concentrations resulted stunted growth of the regenerates while, at higher concentrations, the regenerates showed etiolated growth. None of the lone concentrations could support the formation of multiple shoots and only the combined treatments of different PGRs produced multiple shoots/propagules. The optimum regeneration and multiplication of cultures was accomplished on MS medium containing sucrose (3%), CH (100 mgl^{-1}), CW (15%). Among the different PGRs combinations a combined treatment of NAA-BA (3 + 6 μM

Table 31: Effects of PGRs on plant regeneration and mass multiplication of *C. iridioides*

PGRs Conc. (µM) NAA	IAA	BA	KN	No. of shoot /PLBs formed /explants	Days for formation 1st leaf	1st root	Type of response*
Control				-	-	-	No response
3	-	-	-	4	29	34	Plants healthy
6	-	-	-	2	30	-	Stunted growth
9	-	-	-	2	30	-	As above
	3	-	-	2	80	45	Few PLBs formed
-	6	-	-	2	-	-	As above
-	9	-	-	3	80	-	Stunted small plantlets
-	-	3	-	2	30	-	Plantlets stunted
-	-	6	-	7	30	-	Healthy plantlets with secondary PLBs
-	-	9	-	3	36	-	Few PLBs formed
-	-	-	3	4	16	-	Plantlets with few leaves
-	-	-	6	3	80	56	Stunted growth
-	-	-	9	1	-	-	Small PLB
3	-	3	-	3	16	56	Healthy plants with small leaves
3	-	*6*	-	*20*	*16*	*20*	***Well rooted plantlets with multiple shoot buds/PLBs***
3	-	9	-	6	16	20	Few shoot buds
6	-	3	-	7	80	-	Plantlets etiolated with light green, thin and long leaves.
6	-	6	-	3	80	-	As above
6	-	9	-	2	80	-	As above
9	-	3	-	3	80	-	Stunted growth
9	-	6	-	4	80	-	As above
9	-	9	-	2	80	-	As above
-	3	-	3	2	80	80	Plants stunted
-	3	-	6	2	16	-	Plantlets with small leaves
-	3	-	9	1	59	-	Slightly elongated shoot bud
-	6	-	3	2	-	-	Stunted growth
-	6	-	6	3	80	70	Small plantlets with roots
-	6	-	9	1	-	-	Stunted in growth
-	9	-	3	1	70	-	As above

Contd…

PGRs Conc. (µM)				No. of shoot	Days for formation		Type of response*
NAA	IAA	BA	KN	/PLBs formed /explants	1st leaf	1st root	
-	9	-	6	2	80	-	As above
-	9	-	9	2	80	-	As above
3	-	-	3	5	45	70	Stunted in growth
3	-	-	6	3	16	16	Plantlets slightly etiolated
3	-	-	9	2	70	70	As above
6	-	-	3	4	45	45	Plants healthy with leaves and roots
6	-	-	6	4	45	-	Plantlets healthy
6	-	-	9	3	80	-	Plantlets slightly etiolated.
9	-	-	3	3	80	-	As above
9	-	-	6	2	80	-	As above
9	-	-	9	2	80	-	As above
-	3	3	-	3	16	-	Stunted growth
-	3	6	-	6	45	-	As above
-	3	9	-	3	16	16	Few PLBs
-	6	3	-	2	80	-	Stunted growth
-	6	6	-	2	80	-	Few PLBs formed
-	6	9	-	2	80	-	As above
-	9	3	-	4	45	70	As above
-	9	6	-	4	70	-	Stunted growth
-	9	9	-	3	70	-	Stunted growth

* MS containing sucrose (3%) (w/v), CH (100 mgl^{-1}) and CW (15%) (v/v).

Data represents the mean of three replicates.

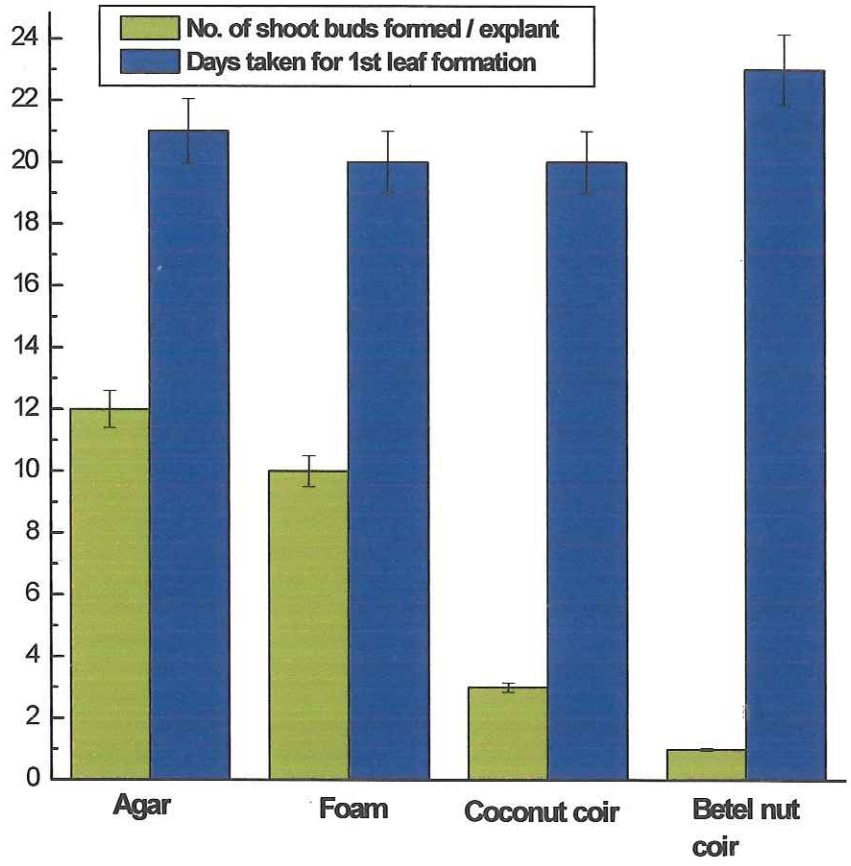

Figure –11

Figure 11: Effects of different substratums on regenerations and mass multiplication of *C. aloifolium* plantlets (*with MS medium containing 3% sucrose, 3 BA μM).*

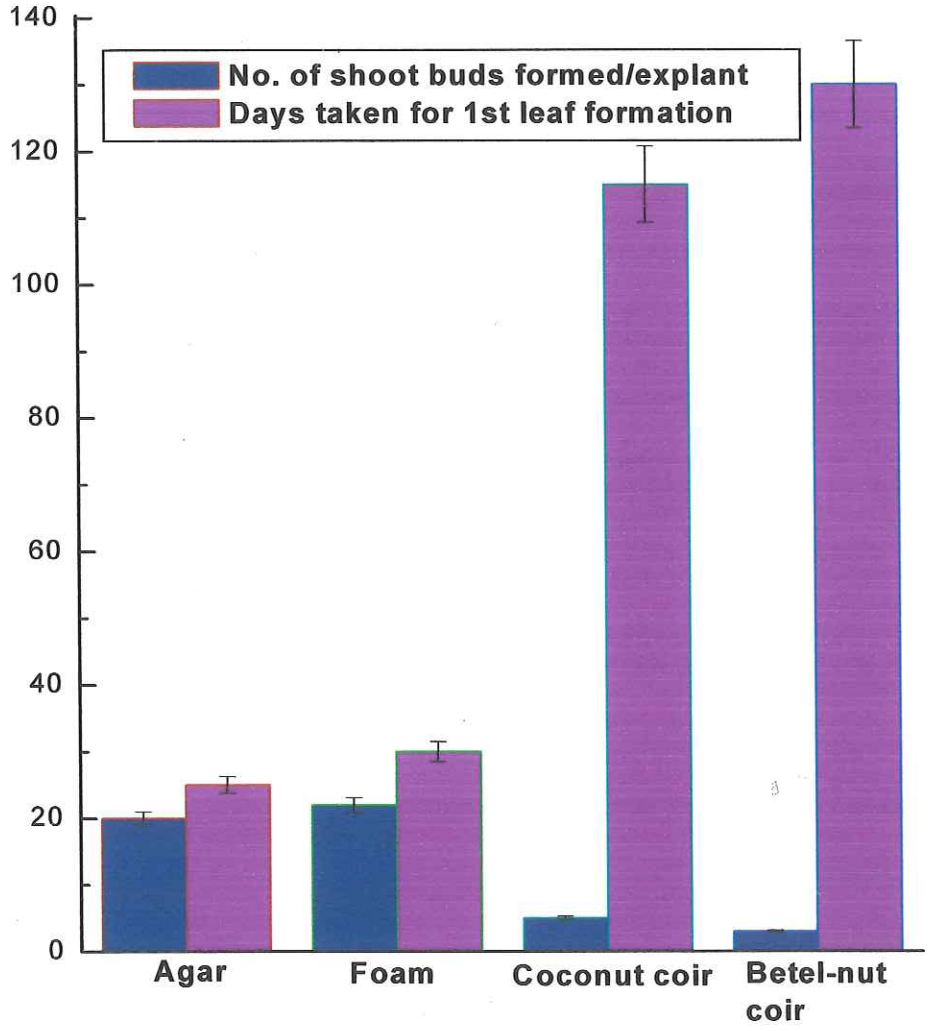

Figure - 12

Figure 12: Effects of different substratums on regenerations and mass multiplication of *C. iridioides* plantlets (*with MS medium containing 3% sucrose, 3 + 6 μM NAA + BA respectively in combination).*

respectively) resulted optimum regeneration and multiplication where as many as 20 plantlets /shoot buds formed after 3-4 wk on regeneration medium (**Fig. 10 a, b, c**).

The performances of cultures on regeneration medium vary with different PGRs combinations. The number of multiple shoot buds formation was significantly higher on MS basal medium containing NAA (3 µM) and BA (6 µM) in combination and plantlets were healthy and well rooted (**Table 31**). But at higher concentrations the regenerates were etiolated and delayed differentiation. In general, the regenerates on medium containing **NAA-KN** in combination supported moderate regeneration and plantlets were healthy and well rooted. The IAA-KN and **IAA-BA** combination of PGRs resulted in fewer shoot buds with stunted growth and poorer root formation.

Effects of Different Alternative Substratums on Regeneration and Mass Multiplication

In the present study, betel-nut coir, coconut coir and foam were successfully used as alternative to agar for germination, regeneration and culture differentiation. In both the species amongst the different substratums incorporated in the regeneration media, better regeneration and multiple shoot buds formation were registered on media containing foam disk as substratum and agar gelled medium (**Fig. 11 & 12**). Of the different substratums, coconut coir and betel-nut coir were found to be inferior compared to agar and foam disk. In *C. aloifolium* as many as 12 shoot buds developed on agar gelled medium against 10 shoot buds on foam disk while, in *C. iridioides* 22 shoot buds developed per subculture on foam disk against 20 shoot buds on agar gelled medium. On media with coconut coir and betel-nut coir as substratums, the number of shoot buds produced was much lower than on agar gelled and foam containing media (**Fig. 11 & 12**). Besides this, cultures maintained on these two conditions required prolonged period for differentiation.

Figure 13: Hardening of regenerated plants and transplanting to community potting mix. **a-b:** ***C. aloifolium*; a.** One of the well rooted regenerated plant in hardening condition; **b.** One of the well hardened plants transplanted to community potting mix; **c-d:** ***C. iridioides*: c.** A rooted plant in hardening condition; **d.** Hardened plants transferred to community potting mix.

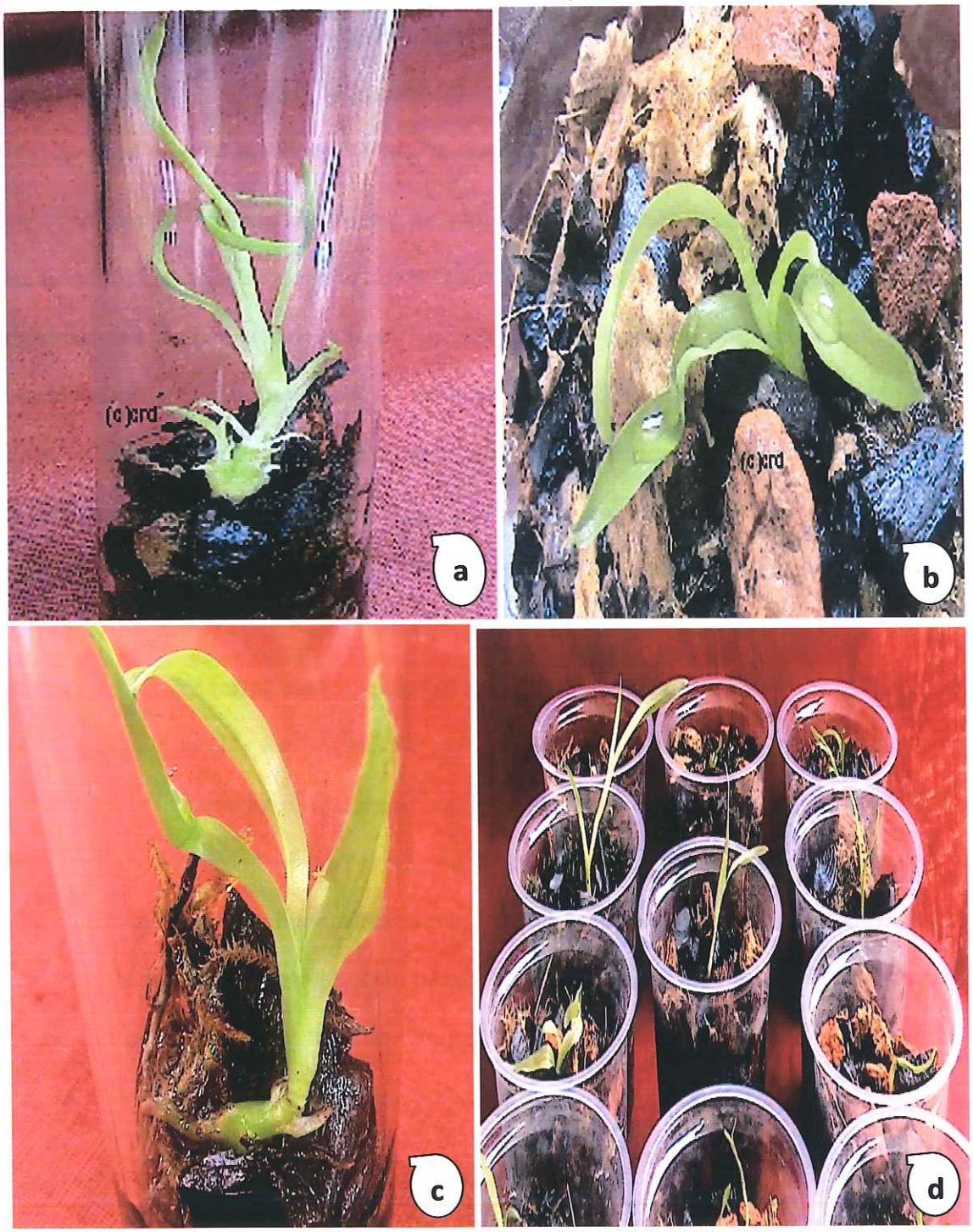

Figure - 13

The well rooted (with 2-3 roots) plantlets of both the species obtained from different explants sources were maintained for 2-3 passages on regeneration medium. The plantlets of ~5-6 cm size were taken out from the regeneration medium and were subjected to *in vitro* hardening before transplanting into potting mix and in the wild.

Hardening and Field Trial of the Regenerates

About 5-6 cm long well-rooted plantlets (with 2-3 roots) of both the species were hardened for considerable period prior to transferring in the potting mix as mentioned in materials and methods (**Fig. 13 a, c**). To transplant the hardened plantlets of both the orchid species (*C. aloifolium* and *C. iridioides*), the CPM was prepared by mixing different substrates like sand: brick pieces: coconut husk: charcoal pieces: decayed wood in different combinations in the ratio of 1:1:1:1 with a layer of moss (**Fig. 13 b, d**). The hardened plants were transferred to CPM along with the contents (substratum of the hardening medium) which were then covered with holed transparent poly bags. The potted plants were maintained in a shaded place and fed with MS liquid salt solution ($1/10^{th}$ strength) weekly for 2-3 wk. The potted plants were exposed to normal day light for about 1 hr in a day for initial one wk and subsequently increased the exposure period to 2 hr from the second wk and finally after one month the plantlets were left in the normal full day light condition which were kept for about 7-8 wk before transferring to the wild. During this process plantlets turned deep green. In *C. aloifolium* ~80% of the transplants survived to form fully developed plants after two months of potting while in case of *C. iridioides* ~75% transplants survived after two months of transfer.

Chapter – 4

Discussions

Initiation of Culture from Immature Embryos/Seeds

A single orchid capsule contains millions of seeds which are microscopic and lack any metabolic machinery. As orchid seeds possess little or no endosperm, their natural germination is limited and the development of seeds in orchid is very poor even at maturity. They need a symbiotic association with specific mycorrhizal fungus which provides an essential physico-chemical stimulus for initiating germination (Harley, 1959). This fungal requirement can, however, be compensated by the *in vitro* incorporation/supplements of sugars/different organic carbon sources and adjuvants. Knudson (1922) demonstrated the possibility of by passing the fungal requirement of orchid seeds during *in vitro* germination and since then asymbiotic/non-symbiotic seed germination has emerged as an important tool for propagating a large number of orchid species and hybrids (Arditti *et al.*, 1982). *In vitro* germination of orchid seeds is an important part of the propagation and conservation programme, as the '*dust seeds*' are tiny and contain limited food reserves. The fertilized ovules/immature embryos are used successfully for micropropagation and rapid mass multiplication of several commercially

viable and or threatened orchids (Devi *et al.*, 1998; Pathak *et al.*, 2001; Sharma and Tandon, 1990; Temjensangba and Deb, 2005a, c). The orchids are also propagated by vegetative means like keikis and or shoot buds.

The successful non-symbiotic seed germination and or immature embryo culture of orchids are greatly influenced by several factors like seed pod age, different nutrient media, different media supplements, plant growth regulators and other culture conditions (Deb and Sungkumlong, 2008, 2009; Deb and Temjensangba, 2006b, 2007b; Sharma and Tandon, 1990; Sungkumlong and Deb, 2008; Temjensangba and Deb 2005a, 2006). However, the media used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the cultured immature embryos/seeds without the intermediary fungus (Mc Kendrick, 2000). But none of these basal nutrient media with different adjuvant fulfil the requirements of the entire orchidaceous group.

In the present study, the asymbiotic seed/immature embryo germination of *Cymbidium aloifolium* and *Cymbidium iridioides* was initiated on five basal media viz., Knudson 'C', Gamborg (B_5), Mitra *et al.*, MS and SH. The immature embryos/green pods of different developmental stages of *C. aloifolium* (7-12 MAP at one month interval) and *C. iridioides* (6-16 MAP at two months interval) were harvested and used for initiation of culture. Nodular swelling was the first sign of germination in both the species with a visible change in colour (circular yellowish embryos) due to physiological changes of cultured embryos followed by greening of the germinating embryos. Then with the passage of time the nodular embryos differentiated into PLBs. The relative time taken for the germination varied with the species. In *C. aloifolium*, the first sign of germination was observed after 25 days of culture while, a similar response in *C. iridioides* was registered after 20 days of culture initiation.

Effects of Basal Media on Immature Seeds Germination

In the present investigation, five different basal media were used for culture initiation from immature seeds/embryos of both the species. Amongst the different media tested, optimum germination was achieved on MS basal medium for both the species. In *C. aloifolium* a germination rate of ~90% was achieved on MS medium followed by Mitra *et al* (70%) and Knudson 'C' (50%) media. Other two media namely B_5 and SH were significantly poorer as these two media did not support germination. A similar trend was also recorded with *C. iridioides* where within 58 days of culture as much as 95% seeds germinated and converted into healthy PLBs on MS medium followed by Mitra *et al,* medium (65%). The orchids of different species exhibit a preferential requirement to specific nutrient media for seed germination but as such no standard media can be prescribed for all the orchid taxa. Deb and Temjensangba (2006b) reported better seed germination of *Malaxis khasiana* on MS medium while, *Arachnis labrosa* on Mitra *et al* medium (Temjensangba and Deb, 2005a), *Cleisostoma racemiferum* on MS medium (Temjensangba and Deb, 2006), *Coelogyne suaveolens* on MS medium (Sungkumlong and Deb, 2008), *Cymbidium macrorhizon* on Mitra *et al* medium (Vij and Pathak, 1988), *Dactylorhiza hatagirea* in Knudson 'C' medium (Vij *et al.*, 1995), *Dendrobium chrysotoxum* in Vacin and Went medium (Rao *et al.*, 1998), *Dendrobium primulinum* on MS medium (Deb and Sungkumlong, 2009), *Eulophia alta* on '*Phyto*Technology Orchid Seed Sowing Medium' (Johnson *et al.*, 2007), *Geodorum densiflorum* on PM medium (Bhadra and Hossain, 2003), *Habenaria macroceratitis* on LC and KC media (Stewart and Kane, 2006), *Rhynchostylis gigantea* on MS medium (Li and Xu, 2009), *Taenia latifolia* on MS medium (Deb and Sungkumlong, 2008), *Vanda coerulea* in Ichihashi & Yamashita (Rao *et al.*, 1998) and VW media (Devi *et al.*, 1998), *Aerides rosea* in Knudson 'C', VW and MS media (Sinha *et al.*, 1998). In the present

study, in *C. aloifolium* SH medium was found to be least suitable while, in *C. iridioides*, Knudson 'C' medium was least preferred for *in vitro* seed germination. A similar response was also reported with other orchid species like *Cymbidiums* (Nagaraju and Upadhyaya, 2001), *Geodorum densiflorum* (Sheelavanthmath *et al.*, 2000) and *Renanthera imschootiana* (Laishram and Devi, 1999).

Effects of Green Pod Age on *In Vitro* Culture of Seeds

A key factor for successful non-symbiotic seed germination in both the species was the physiological age of the green pod/capsule. Different species of orchids exhibit a particular threshold, a factor genetically structured in the organism. The influence of physiological age varies with the genus, species within the genus. There is a window period of seed development for every orchid species, which supports optimum *in vitro* germination. The earliest stage at which the embryos can be cultured successfully varies within the orchid genotype and local conditions (Deb and Temjensangba, 2006b; Sauleda, 1976; Temjensangba and Deb, 2005a). Therefore it is desirable to determine the right stage to harvest the green capsule/pods to achieve an optimal germination. The culture of immature green pod/embryos ensures sterility but may require prolong period for germination as the seeds are immature. While, the culture of comparatively mature capsules/embryos before dehiscing may support better germination but the chances of contamination increases due to establishment of mycorrhizal association (Mc Kendrick, 2000). In the present investigation, the green pod age of 9 MAP supported optimum germination (90%) in *C. aloifolium* while in *C. iridioides;* the optimum germination was achieved from green pod of 10 MAP where about 95% germination was recorded. The green pod age <8 MAP in *C. aloifolium* and <6 MAP in *C. iridioides* either failed to germinate or germinated after prolonged period of culture. In *Dacylorrhiza hatagirea*, seeds of 16 WAP (Vij *et al.*, 1995), in *Malaxis khasiana*, seeds of 8 WAP (Deb and

Temjensangba, 2006b), in *Rhynchostylis gigantea*, 4 months old seeds (Li and Xu, 2009) exhibited better germination but 12 WAP was ideal planting materials in *Cymbidium macrorhizon* (Vij and Pathak, 1988). The ability of immature embryos to germinate better than the mature ones is due to their distended testa cells and metabolically awakened embryos and lack of dormancy and inhibitory factors (Yam and Weatherhead, 1988).

Effects of Organic Carbon Source, CH and CW on A-symbiotic Seed Germination

For *in vitro* culture of immature embryos of various developmental stages, three different organic carbon sources (viz. Dextrose, glucose and sucrose) were tested at different strengths. It was observed that incorporation of at least one of the organic carbon sources was pre-requisite for successful *in vitro* culture of immature embryos/seeds of both the selected species. In absence of organic carbon source, only nodular swellings of embryos were observed in both the species (Table 5 & 6). The swelled seeds failed to convert into PLBs and degenerated subsequently. Presence of organic carbon sources such as dextrose, glucose and sucrose in the initiation media showed a marked effect on seeds germination. The different levels of the organic carbon sources showed differential effect on seed germination. The requirements of the quality and quantity of exogenous supply of the organic carbon sources vary with the species, the media compositions used, the endogenous level of organic carbon and the developmental stage of the cultured embryos (Temjensangba and Deb, 2005c). Sharma and Tandon (1990) reported that 2-3% sucrose, D-Fructose and D-Glucose were the suitable organic carbon sources for *in vitro* seed germination of *Cymbidium elegans* and *Coelogyne punctulata* while, 3% sucrose was found to be suitable for immature seed germination of *Geodorum densiflorum* (Bhadra and Hossain, 2003). In the present study, amongst the different organic carbon sources tested, optimum seed germination of *C.*

aloifolium and *C. iridioides* was obtained on basal media containing 2% sucrose. With *C. aloifolium* glucose was found to be inferior in its entire range tested for seed germination as it delayed germination and failed to support healthy PLBs formation (Table 5). While, dextrose was least suitable for seed germination and subsequent differentiation in *C. iridioides* compared to the other two organic carbon sources (Table 6).

In the present study for *in vitro* immature embryo culture, incorporation of either CW or CH had little effect on germination as well as subsequent differentiation in both the species. In the past many workers have used CW and CH in the germination media. The stimulatory effect of CW was reported in *Arachnis labrosa* (Temjensangba and Deb, 2005a), in *C. racemiferum* (Deb and Temjensangba, 2007b), in *C. suaveolens* (Sungkumlong and Deb, 2008), in *Dendrobium aphyllum* (Talukdar, 2001), in *Taenia latifolia* (Deb and Sungkumlong, 2008), in *Vanda coerulea* (Devi *et al.*, 1998). Better seedling differentiation was achieved in *Peristeria elata* when the medium was supplemented with CW (10%) and proved to be advantageous over peptone (Bejoy *et al.*, 2004) while, Sinha *et al.* (2002) and Vij and Aggarwal (2003) reported the enhanced repetitive PLBs formation in Renades and *Vanda coerulea* respectively when media were supplemented with CW (15%). However, Sheela *et al.* (2004) reported a rapid multiplication of PLBs as well as the development of shoots in *Dendrobium* CV Sonia when media is supplemented with 7.5% CW in the presence of BA (1.5 mgl^{-1}). Leetham (1974) reported that a plant growth regulator like substance (cytokinins) is present in coconut water whereas Lee *et al.* (1995) attributed its effect to its sugar and cytokinin content.

Effects of Light Intensity on A-symbiotic Seed Germination

In the present study with the two selected orchids different light intensity exhibited differential effect on nodulation of seeds, per cent germination and time for

PLBs formation. In both *C. aloifolium* and *C. iridioides*, cultures maintained in dark exhibited early nodulation but, optimum germination (90% in *C. aloifolium* and 95% in *C. iridioides*) was registered under full light (40 µmol m^{-2} s^{-1}) compared to cultures maintained in the dark and diffused light (20 µmol m^{-2} s^{-1}) conditions. In *Arachnis labrosa* (Temjensangba and Deb (2005a) and in *C. racemiferum* (Deb and Temjensangba, 2007b, Temjensangba and Deb, 2006), diffused light condition of 20 µmol m^{-2} s^{-1} was found suitable for initiation of asymbiotic germination followed by full light (40 µmol m^{-2} s^{-1}) conditions at 12/12 hr photoperiod while, in *Habenaria macroceratitis* (Stewart and Kane, 2006) 8/16 hr (L/D) photoperiod was preferred over 0/24 and 12/12 hr photoperiod. But, Deb and Temjensangba (2006b) observed that diffused light condition supported higher rate of germination in *Malaxis khasiana*. Islam *et al.* (2003) reported that the *Phalaenopsis* callus growth and development of PLBs were better in illumination than in dark. The effect of light for embryogenic culture initiation in conifers is well documented (Deb and Tandon, 2004; Gupta and Grob, 1995; Von Arnold, 1987). In *Pinus kesiya*, light was reported inhibitory for initiation of embryogenic cultures and produced more non-embryogenic cultures. Dark was optimum while diffused light formed moderate embryogenic cultures (Deb and Tandon, 2004).

Effect of PGRs on A-symbiotic Seed Germination

In the present investigation, even in the absence of PGRs, nodular swellings of the immature embryos were possible but failed to differentiate further. The PGRs in the germinating medium showed a marked effect on the growth, differentiation and development of PLBs. The observation in the present study is in agreement with the observation made by Deb and Temjensangba (2006b), Temjensangba and Deb (2005a), and Li and Xu (2009). In the present study, seed germination of *C. aloifolium* was recorded as high as ~90% on MS medium containing NAA-BA (3 + 6 µM respectively)

in combination but in *C. iridioides* optimum seed germination (~95%) was recorded on MS medium containing NAA-BA (3 + 3 µM respectively) in combination. The responding seeds swelled and converted into PLBs on initiation medium after 58 days in both the species. A synergistic action of auxin and cytokinin was observed in asymbiotic seed germination in the present investigation. The present observation is in agreement with Sharma and Tandon (1986); Li and Xu (2009) where they reported the stimulatory effect of NAA in conjunction with cytokinins. In *Rhynchostylis gigantea* NAA (0.2 mgl^{-1}) and BA (0.05 mgl^{-1}) supported optimum germination. But Nagaraju *et al.* (2004) reported that in *Dendrobium* hybrid, the single leaflet in the PLB developed when basal medium was supplemented by BAP alone while, BA singly supported better germination in *Malaxis khasiana* (Deb and Temjensangba, 2006b).

Effect of Different Alternative Substratums on A-symbiotic Seed Germination

Apart from agar, other materials like betel-nut coir, coconut coir, foam disk and leaf litter could be successfully used with differential success for seed germination. In *C. aloifolium* foam containing medium supported higher seed germination (90%) against agar gelled medium (80%) followed by coconut coir (75%), betel-nut coir (65%) and leaf litter (50%) containing medium. While, in case of *C. iridioides,* germination rate was slightly higher (~95%) on agar gelled medium compared to foam (85%), betel-nut coir (90%), coconut coir (70%) and leaf litter (50%) (Table 13 & 14). Though in *C. iridioides,* germination rate was higher on agar gelled medium, it required longer duration compared to other substratum. Agar-gelled medium supported germination only after 58 days of culture while within 43 to 49 days embryos germinated on media with other substratum. A similar trend was also observed with *C. aloifolium.* Besides the time taken for germination and per cent germination, subsequent differentiation was equivalent to that achieved on agar and in some cases the alternative substratum out

performed agar. Amongst the different substratum studied in the present investigation, better germination and subsequent differentiation were registered on medium containing 'Foam' followed by 'Coconut coir, 'Betel-nut coir' and 'Leaf litter'. On medium containing 'betel nut coir' and 'coconut coir' as substratum, it supported moderate germination but delayed differentiation, and cultures on chopped leaf litter failed to differentiate properly.

Initiation of Culture from Leaf, Aerial Root and Nodal Explants

In the current investigation cultures were also initiated from the foliar explants, aerial roots and nodal explants of both the species from *in vitro* source. Wimber (1965) successfully developed PLBs from the leaves of *Cymbidiums*, which opened up an effective alternative to apical shoot meristem culture. Since then the regenerative competence of foliar explants were positively tested for more than 60 orchid species (Temjensangba and Deb, 2005b). However, the success is restricted mostly with epiphytic orchids and only few species from terrestrial orchids suggesting thereby that the ground orchids are less amenable to *in vitro* regeneration (Deb and Sungkumlong, 2010). In the present study, initiation of morphogenetic response was restricted to the basal parts of the foliar explants in both the species. The morphogenetic potential of leaf base has been reported in *Coelogyne, Dendrobium, Oncidium* and *Phalaenopsis* (Abdul Karim and Hairani, 1990), *Acampe praemorsa* (Nayak *et al.*, 1997), *A. Labrosa* (Deb and Temjensangba, 2007a), *C. racemiferum* (Temjensangba and Deb, 2005b), *C. suaveolens* and *Taenia latifolia* (Deb and Sungkumlong, 2010), *V. coerulea* (Vij and Aggarwal, 2003). Mathews and Rao (1985) considered the leaf base to be the decisive factor for culture initiation from foliar explants. Sinha and Hegde (1999) reported the development of meristematic activity along the entire leaf in Renades Arunoday Hybrid. In the present study the orientation of explants was observed to be another crucial factor for

morphogenetic response in both the species. Slanted orientation (~45° angle) of the explants showed better response than horizontally placed explants. Earlier, Nayak *et al.* (1997) in *Acampe praemorsa* reported the influence of explants orientation of shoot development.

The incorporation of PGRs to the basal medium was obligatory for the initiation of culture. The explants of both the orchid species failed to respond, when cultured on PGRs free medium. The role of growth hormones in stimulating meristematic activity and promoting proliferation in leaf explants is well documented in orchids (Abdul Karim and Hairani, 1990; Arditti and Ernst, 1993; Deb and Sungkumlong, 2010; Deb and Temjensangba, 2007a; Li and Xu, 2009; Nayak *et al.*, 1997; Temjensangba and Deb, 2005b; Vij and Pathak, 1990; Vij *et al.*, 1994; Yam and Weatherhead, 1991). Murashige (1974) opined that *in vitro* plant regeneration occurs frequently through adventitious shoot formation and rarely through somatic embryogenesis. In *C. aloifolium* meristematic loci invoked after about 65 days of culture initiation where as many as 10 shoot buds, PLBs formed per explants in about 52% of cultures (Table 15). Amongst the different PGRs tested, optimum response was registered on MS medium containing sucrose (3%), NAA and BA (6 and 9 µM respectively in combination). With *C. iridioides*, ~90% cultures responded positively after 90 days of culture where 20 shoot buds developed at the basal part of the foliar explants on medium containing NAA and BA (9 and 3 µM respectively) in combination (Table 16).

In the present study, the root explants of both the selected orchid species were cultured on MS basal medium and the explants failed to regenerate in the absence of PGRs. About 5-6 wk old aerial roots of *C. aloifolium* and *C. iridioides* from axenic culture responded positively on MS medium enriched with sucrose (3%). In case of *C. aloifolium*, of the different PGRs used in the present investigation, ~60% explants

responded positively after 20 days of culture on MS medium containing sucrose (3%) and KN (3 μM) where shoot buds and PLBs formed at both ends of the root (Table 17). Both the auxin sources (IAA and NAA) used singly impaired regeneration. When IAA was used in conjunction with KN, it improved the morphogenetic response significantly, but NAA used in combination with BA did not improve the response. While, in case of *C. iridioides*, cultured aerial roots remained recalcitrant on different PGRs enriched medium unless AC was incorporated in the medium. At lower concentration of AC, only elongation of roots observed. The meristematic loci were invoked only when MS medium was enriched with AC (0.1%, w/v) where swellings of root tips followed by PLBs formation were observed after 40 days of culture (Table 18). At higher concentration of AC, cultures degenerated without differentiation. MS basal medium containing sucrose (3%), AC (0.1%) and IAA (3 μM) was recorded optimum (50%) for the morphogenetic response of aerial roots of *C. iridioides*.

In the present study, KN (3μM) supported *in vitro* morphogenetic response in *C. aloifolium* and IAA (3μM) supported optimum morphogenetic response in *C. iridioides*. But in *A. labrosa* IAA-KN (4 μM + 6 μM respectively) in combination resulted shoot buds formation in ~ 80% cultures (Deb and Temjensangba, 2006a) while in *C. racemiferum* root (~15-20 wk old) from axenic culture exhibited 100% meristematic activity on basal medium containing sucrose (3%), CH (200 mgl^{-1}) and IAA-Kn (1 μM each) in combination enriched with sucrose (3%) (Deb and Temjensangba, 2005). However, Vij *et al.* (2004) reported that prolonged cultures in the medium containing NAA proved useful for PLBs mediated culture multiplication in *Cymbidium* root and combined treatment with NAA and BAP induced direct callus mediated development and enhanced response frequency in root explants. Philip and Nainer (1988) observed that the requirement of exogenous supply of PGRs is species specific and it varies during

initiation, multiplication and differentiation of cultures. The transformation of root meristem to shoot meristem is positively influenced by the endo- and or exogenous level of auxin. In another report by Peres *et al.* (1999) observed that the exogenous PGRs markedly influenced the morphogenesis by alternation in the endogenous IAA/cytokinin balance.

Cultures were also initiated with nodal segments of both the species from *in vitro* source. In *C. aloifolium* sprouting of the shoot buds/PLBs from the nodal region of the segments were initiated. Morphogenetic response was observed from the nodes within 15 days of culture. A single treatment with NAA was more effective over BA for culture differentiation. In about 90% cultures ~ 5 shoot buds/PLBs developed per explants at a single node on MS medium supplemented with sucrose (3%) and NAA (9 µM). From the nodal segments of *C. iridioides* optimum response was recorded on MS medium fortified with sucrose (3%) and NAA (3 µM) where as many as 6 shoot buds/PLBs were developed from a node after 4 days of culture (Table 21). Positive response was recorded with both single and combined treatments of NAA and BA. Though per cent response was higher with combined treatments, the number of shoot bud formation was higher with single treatment of NAA (3µM) where shoot buds invoked within 4 days of culture.

In the present study with the two selected species of *Cymbidium* NAA was found to superior over BA for morphogenetic response. But in *Phalaenopsis* BA (2 mgl^{-1}) and NAA (0.5 mgl^{-1}) in combination was found to be optimum for breaking axillary buds and formation of multiple shoot buds (Kosir *et al*, 2004) and observed that incorporation of NAA was promontory. Other workers like Tisserat and Jones (1999), Roy and Banerjee (2003) also observed that an appropriate combination of NAA and BA stimulated multiple shoot buds formation. But, Arditti and Ernst (1993) reported that the addition of NAA reduced induction and regeneration.

Regeneration of Plantlets and Mass Multiplication

The PLBs and shoot buds formed from the cultured immature embryos/seeds, foliar explants, aerial roots, nodal segments of both the species were maintained for another 2 passages for further differentiation and proliferation on optimum initiation conditions. The advanced stage PLBs, shoot buds developed from the germinated seeds, foliar explants, aerial roots and cultured nodal explants were subjected to regeneration of plantlets and mass multiplication.

Effect of Basal Media on Regeneration and Mass Multiplication

In the preliminary study, the PLBs, shoot buds and tiny plantlets of *Cymbidium aloifolium* and *Cymbidium iridioides* were maintained on three different basal media viz., Knudson 'C', Mitra *et al* and MS media in conjunction with different adjuvants for regeneration of plantlets and mass multiplication. The PLBs/shoot buds differentiated into plantlets after 2-5 wk on transferring to a regeneration medium. Amongst the three media tested, it was found that MS medium supported optimum growth and differentiation into rooted plantlets in both the species under study compared to the other two media, which supported poorer culture and growth, differentiation and proliferation.

Further experiments were conducted to study the effect of different strengths of MS medium on regeneration of plantlets and culture proliferation. It was found that full strength of MS medium supported the formation of maximum shoot buds and well-rooted healthy plantlets in both the species. In *C. aloifolium*, most of the plantlets were either stunted in growth or etiolated at lower strengths (0,1/4th, ½, 3/4th) with the formation of fewer shoot buds as compared to the number of shoot buds formed in full MS strength (Table 24). Similarly, in *C. iridioides*, full strength of MS medium supported better regeneration where as many as 20 shoot buds were formed. At lower strengths (0, 1/4th, and ½) of MS medium, only few shoot buds were formed and the

plantlets were stunted in height. While at 3/4th strength of MS medium, though fewer shoot buds were formed, the plantlets regenerated were healthy with well developed roots and leaves (Table 25). This perhaps could be due to difference of chemical constituents with MS medium or deficient as to the requirement of the developing seedlings. Chen *et al* (2004), George and Sherrington (1984), and Temjensangba and Deb (2005a) argued that the change in culture conditions and media could alter the pattern of organogenesis in orchids and such behaviour can be judiciously exploited to achieve desirable response in many orchid taxa by altering the nutrient regimen.

Effect of Different Organic Carbon Sources on Regeneration and Mass Multiplication

Various organic carbon sources such as dextrose, glucose and sucrose (0-4%) were incorporated in the regeneration medium where sucrose rich medium was found to be preferable by both the species. Incorporation of at least one of the organic carbon sources was obligatory for regeneration and mass multiplication of plantlets. There was no regeneration in the absence of the organic carbon sources (Table 26 & 27). The requirements of the quality and quantity of exogenous supply of the organic carbon sources varies with the species, the media compositions used, the endogenous level of organic carbon and the developmental stage of the cultured embryos (Temjensangba and Deb, 2005a, c). Sharma and Tandon (1990) reported that 2-3% of sucrose; D-Fructose and D-Glucose were the suitable organic carbon sources for *in vitro* seed germination of *Cymbidium elegans* and *Coelogyne punctulata*. In the present investigation, amongst the different organic carbon sources tested, optimum regeneration and shoot bud formation in *C. aloifolium* were obtained on basal media containing sucrose (3%) where as many as 12 shoot buds were formed per subculture. The media enriched with dextrose supported fewer shoot bud formation (3-8) whereas, glucose enriched media did not

support regeneration and degenerated subsequently. With *C. iridioides* dextrose was found to be inferior as it failed to support formation of healthy plantlets and shoot buds as compared to the media fortified with glucose which supported moderate regeneration. As many as 6 shoot buds resulted in media containing a lower concentration of glucose (1%). A higher concentration of sucrose was registered to be better for faster culture differentiation and proliferation (Table 27). Optimum regeneration and culture multiplication were achieved on MS medium containing sucrose (3%) where as many as 20 shoot buds formed per plant per subculture.

Effect of CH and CW on Regeneration and Mass Multiplication

Different concentrations of CH (0-200 mgl^{-1}) and CW (0-20%) were added in the regeneration medium either singly or in combination. In both the species, though incorporation of CH and CW resulted in healthy plantlets, most of the plantlets were either stunted in growth or etiolated. Addition of CH (100 mgl^{-1}) and CW (15%) singly or CH (100 $mgl^{-1)}$ and CW (10%) in combination in *C. aloifolium* supported the formation of 10 shoot buds per explant and healthy but stunted plantlets. In rest of the treatments either with CH or CW singly, or in combination, it had little effect on multiple shoot bud formation and regeneration. In case of *C. iridioides*, presence of CH in the medium had no promotory effect and the number of shoot buds formed were either lesser or at par when compared to the control. While, the incorporation of CW (15%) in the media resulted in the formation of as many as 20 shoot buds per explant in just about 14 days of culture. But though the plantlets were healthy with well-developed roots and leaves, they were slightly pale green in colour when compared to the plantlets resulted with CH which were darker green in colour. No significant effect was observed in rest of the lone treatments of CW. A synergistic effect of CW and CH on formation of shoot buds and healthy plantlets were achieved on medium containing a combined treatment of

CH (100 mgl^{-1}) and CW (15%) where as many as 20 shoot buds per explants were formed and exhibited dark green healthy plantlets.

The stimulatory effect of CW was reported in *Arachnis labrosa* (Temjensangba and Deb, 2005a), in *C. racemiferum* (Deb and Temjensangba, 2007b), in *C. suaveolens* (Sungkumlong and Deb, 2008), in *Dendrobium aphyllum* (Talukdar, 2001), in *Taenia latifolia* (Deb and Sungkumlong, 2008), in *Vanda coerulea* (Devi *et al.*, 1998). Better seedling differentiation was achieved in *Peristeria elata* when medium was supplemented with CW (10%) and proved to be advantageous over peptone (Bejoy *et al.*, 2004) while, Sinha *et al.* (2002) and Vij and Aggarwal (2003) reported the enhanced repetitive PLBs formation in Renades and *Vanda coerulea* respectively when media were supplemented with CW (15%). However, Sheela *et al.* (2004) reported a rapid multiplication of PLBs as well as the development of shoots in *Dendrobium* CV Sonia when media was supplemented with 7.5% CW in the presence of BA (1.5 mgl^{-1}). Leetham (1974) reported that a plant growth regulator like substance (cytokinins) is present in coconut water whereas Lee *et al.* (1995) attributed its effect to its sugar and cytokinin content.

Effect of PGRs on Regeneration and Mass Multiplication

Different quality and quantity of PGRs marked a pronounced effect and elicit different responses in the seedling development. Inclusion of PGRs in the regeneration medium was obligatory for successful regeneration of plantlets and mass multiplication. In the absence of PGRs there were only few regenerates in *C. aloifolium* whereas in *C. iridioides*, cultures remained recalcitrant and degenerated subsequently. In both the species, both the auxins (IAA and NAA) when used singly either impaired regeneration or failed to produce healthy plantlets compared to cytokinins (BA and KN). Of the two

auxins used, IAA was found to be inferior over NAA in both the species (Table 30 & 31).

In *C. aloifolium*, the optimum response in terms of plantlet regeneration and mass multiplication was achieved on regeneration medium containing a single treatment of BA (3 µM), where as many as 12 shoot buds/PLBs were formed per explant and the first leaf and root were observed just after 21 days of culture (Table 30). Of the four different PGRs tested, NAA and IAA singly did not support healthy regeneration and in most of the cases either plants were etiolated or fewer shoot buds were formed. While, lone treatments of both BA and KN supported better regeneration and culture proliferation and BA was found to be superior over KN. Though NAA or IAA singly did not support healthy culture differentiation, combined treatments of NAA + BA were found to be beneficial. A combined treatment of NAA + BA (6 + 3 µM respectively) exhibited faster culture differentiation compared to single treatment with BA where first leaf was released after 13 days of culture initiation (Table 30). But combined treatments of IAA and KN were found to be inhibitory and failed to support regeneration as well as culture proliferation and in most of the cases cultures failed to produce roots. Other combination like NAA + KN and IAA + BA did not support any regeneration and cultures degenerated subsequently.

In *C. iridioides*, the advanced stage PLBs from the cultured seeds started converting into young rooted plantlets and repetitive PLBs within 3-4 wk on regeneration media. It was observed that the single treatment with different PGRs at lower concentrations resulted stunted growth of the regenerates while, at higher concentrations the regenerates showed etiolated growth. None of the concentrations could support the formation of multiple shoots and only the combined treatments of different PGRs produced multiple shoots/propagules. The optimum regeneration and

multiplication of cultures were accomplished on MS medium containing sucrose (3%), CH (100 mgl^{-1}), CW (15%). Among the different combinations of PGRs, a combined treatment of NAA-BA (3 + 6 µM respectively) resulted optimum regeneration and multiplication where as many as 20 plantlets /shoot buds formed after 3-4 wk on regeneration medium (Table 31).

The inhibitory effect of IAA on seedling development has been reported in *Orchis purpurella* (Hadley and Harvais, 1968), *Dactylorhiza purpurella, Coeloglossum viride* and *Platanthera bifolia* (Hadley, 1970). On the other hand, IAA at 0.1 mgl^{-1} is reported to promote seed germination and seedling development of *Cymbidium punctulata* (Sharma and Tandon, 1986). But Vij and Aggarwal (2003) reported that NAA favoured the development of multiple shoots/PLBs in *Vanda coerulea*. Bhadra and Hossain (2004) reported highest number of multiple shoot buds formation from nodal segment of *Micropera pallida* when medium was supplemented with 2.0 mgl^{-1} NAA and 2.0 mgl^{-1} BA. While the PGRs like BAP singly or in combination with IAA were best used for initiation of cultures and development of healthy plantlets from leaf explants of *Saccolabium papillosum* (Kaur and Vij, 2000).

Effect of Alternative Substratums on Regeneration and Mass Multiplication

The germinating seeds on different substratum converted into PLBs. The PLBs formed on germination medium were transferred to full light condition where they turned green and started differentiating. The advanced stage PLBs/shoot buds were also maintained on the regeneration medium containing different alternative substratums such as betel nut coir, coconut coir and foam besides agar. Within 2-3 weeks of culture on regeneration medium the advanced stage PLBs started differentiating into rooted plantlets and multiple shoot/buds. For culture differentiation as well as proliferation, foam supported medium was found to be superior over other substratums and agar gelled

medium. In *C. aloifolium,* amongst the different substratums incorporated in the optimum regeneration medium, better regeneration and multiple shoot bud formation were registered on medium containing foam as the substratum where as many as 10 shoot buds were formed. Fewer shoot bud and smaller plantlets were observed on medium containing coconut coir and betel nut coir as substratum which also took longer duration for the development of leaves and roots. In case of *C. iridioides*, amongst the different substratum used, agar gelled medium and foam incorporated medium supported similar regeneration and culture proliferation compared to the other two substratums (coconut coir and betel nut coir) supported media.

It was observed that the initial response was better on agar gelled medium as cultures establish on this medium faster compared on the other substratums. However, once the cultures establish themselves on the alternative substratums especially on foam and coconut coir, they exhibited healthier growth and rapid culture proliferation compared to cultures on agar gelled medium.

During the last two decades, a number of substances viz., agarose (Johansson, 1988), alginates (Scheurich *et al.*, 1980), gelrite (Pasqualetto *et al.*, 1988), guar gum (Babbar *et al.*, 2005; Jain *et al.*, 2005), isubgol (Babbar and Jain, 1998; Jain *et al.*, 1997), starch (Zimmerman *et al.*, 1995; Nene *et al.*, 1996), xanthan gum (Babbar and Jain, 2006) etc, have been used with reasonable success as substitutes of agar. However, these are not expected to find universal acceptance for various reasons. Alginates gel only in the presence of specific ions and therefore are not suitable substitute of agar in many circumstances, while agarose is cost prohibitive. Starch has inferior gelling ability, poor clarity as well as a metabolizable nature which can result in softening of the media. Isubgol, due to its polysaccharide nature, good gelling ability, gel clarity and resistance to enzymatic activity, has the potential to become a universal gelling agent for plant

tissue culture media, but due to its high melting point (~70°C) it needs *p*H adjustment and fast dispensing (Babbar and Jain, 2006).

Effect of Substratum and Cost Effectiveness

In the present study different substratums (viz. betel-nut coir, coconut coir, foam and forest leaf litter) were used as alternative to agar for germination, regeneration and mass multiplication. However, foam supported culture outperformed coconut coir, betel-nut and leaf litter supported culture in all the three stages i.e., germination, plant regeneration and multiplication. It was observed that the initial response for germination and plant regeneration was superior on agar gelled medium as cultures establish on this medium faster compared to other substratums studied. But once the culture gets established on alternative substratum especially on 'foam' and 'coconut coir', the cultures exhibit healthier growth and rapid culture proliferation compared to agar gelled medium. This conclusion is based on the following observations/facts:

I. The embryos germinated on agar gelled medium after about 58 days of culture in both the species followed by differentiation into healthy green PLBs. On regeneration medium gelled with agar as many as 12 and 20 shoot buds (*C. aloifolium* and *C. iridioides* respectively) formed per sub-culture per PLB.

II. While, seeds cultured on foam as supporting material in otherwise identical condition, required 45 days for germination in both the species. On regeneration medium with foam as the substratum, about 10 shoot buds in *C. aloifolium* and 22 shoot buds in *C. iridioides* were formed from a single propagule which differentiated into dark green healthy plantlets, about 5-6 cm long with broad leaves and roots.

III. Seeds cultured on coconut coir-supported medium germinated after 43 days of culture. Though the PLBs were smaller in size and differentiation was delayed compared

to agar-gelled and foam-supported media, it resulted into dark green and healthy plantlets.

The goal of the present study was to screen some low cost substratums as alternatives to agar for use in orchid tissue culture so that the production costs could be reduced substantially. In the present study, it was recorded that in agar gelled medium, agar constitutes about 25% of the media cost excluding plant growth regulators. Presently in plant tissue culture one of the costliest ingredients is gelling agent and in most of the cases it is agar which makes the tissue culture a costly technique. If we wish to make the plant tissue culture technique more users friendly and incorporate in floriculture/horticulture and conservation programmes, alternative substratums to agar is/are desirable. One way to reduce the production cost is the use of low cost substratums such as 'betel-nut coir, coconut coir, foam, and leaf litter' as these materials have certain advantages over agar or other gelling agent. Agar and other comparatively cheaper gelling agents like phytagel need to be incorporated every time we prepare the medium as they dissolve in the medium and is discarded in every sub-culture. But materials like foam, coconut coir, betel-nut coir and forest litters can be recycled and same materials can be used many times.

The hunt for the cheap substratum could be that which is/are of very low cost. In the past some efforts have been put into to use other low cost substratum to replace agar. In most of the cases success was restricted to initiation of culture only. Aggarwal *et al.* (2006) reported the use of coconut coir as substratum for seed germination of *Cymbidium pendulum* and success was restricted to germination stage only. Deb and Temjensangba (2006b), Deb and Imchen (2010), Temjensangba and Deb (2005a, 2006) could successfully use forest litter and moss as substratum in the hardening medium. In the present study betel nut coir, coconut coir, and polyurethane foam disk could be

successfully used as alternative to agar for germination, regeneration etc. But better germination, regeneration of plantlets and multiple shoot bud formation were registered on medium containing foam disk compared to other substratums. It was observed that the initial response for germination, regeneration was better on agar gelled media, as the cultures establish on this media faster compared to the alternative substratum containing media. But once the culture establish on alternative substratum especially on 'foam disk' and 'coconut coir', the cultures exhibit healthier growth and rapid culture proliferation compared to agar gelled medium. According to estimation in the present study, the overall production cost could be successfully reduced to ~25% compared to agar gelled culture. This distinct cost advantage is mostly due to:

(i) The agar gelled cultures demand subcultures at every 3-4 wk on fresh medium which many a times invite unwanted microbial contamination. But with the cultures on alternative substratum this problem could be ruled out as the fresh medium can be poured in the same culture vials at regular interval and only the proliferated propagules are transferred to fresh culture vials. Besides this, it cuts the manpower costs.

(ii) As most of the substances used in the present study are either the wastes of house holds or are very cheap.

iii) These substratums are recyclable e.g., if there is contamination, the substratums can be washed, autoclaved and reused unlike agar.

Apart from costs effectiveness, use of these substratums as in the present study does not exert pressure on nature and environment as most of the substratums are natural and renewable sources except 'foam' which is synthetic. Their increased demands can be met without any fear of exploitation of its resources and also does not pose much threat to environment on being disposed after use. It is further observed that the cultures maintained on alternative substratum establish better in the community potting mix

compared to the cultures maintained on agar gelled medium as in most of the cases the roots of the regenerates adheres to the substratum as does by velamenous roots of orchids in their natural habitats.

Hardening and field trials of the regenerates

The hardening of in *vitro* raised plantlets is essential for better survival and successful establishment. In other words the survival percentage is determined by the hardening of the plantlets. Losses of micropropagated plants after transferring to nature are attributed to low humidity, high levels of light and non-sterile condition of the *in vivo* environment (Deb and Imchen, 2010, Lavanya *et al.*, 2009; Paul, 1999). Conventionally the tissue-raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at high light intensity. Different matrix or substrates with manipulation in salt solution were employed for hardening of different angiospermic *in vitro* raised plants by various workers viz: soilrite for *Carica papaya* (Agnihotri *et al.*, 2004), soaked cotton for *Saccharum officinarum* (Gill *et al.*, 2004) etc.

During the present investigation, the well rooted healthy plantlets of both the species were removed from the regeneration medium and transferred into culture vials containing $1/10^{th}$ strength of MS salt solution supplemented with 1 % (w/v) sucrose devoid of any PGR as described by Deb and Imchen (2010). In each culture tube different types of supporting materials such as charcoal pieces, brick pieces, and chopped mosses (at 1:1 ratio) were incorporated before the plantlets are transferred. The cultures were then maintained for ~6-7 wk under identical culture conditions. The hardened plants were then transferred to community potting mix-containing sand: brick pieces: coconut husk: charcoal pieces: decayed wood in different combinations in the ratio of 1:1:1:1 with a layer of moss which were covered with holed transparent poly-bags. The potted plants were maintained in a shaded place and fed with MS liquid salt solution

($1/10^{th}$ strength) for the initial 2-3 wk. The potted plants were exposed to normal day light for about 1 hr in a day for initial one wk and subsequently increased the exposure period by 2 hr from the second wk and finally after one month the plantlets were left in the normal full day light condition which were kept for about 7-8 wk before transferring to the wild. During this process plantlets turned deep green. In *C. aloifolium* ~80% of the transplants survived to form fully developed plants after two months of potting while in case of *C. iridioides* ~75% transplants survived after two months of transfer. Feeding the plantlets with nutrient salt solution has been reported to be beneficial for the promotion of orchid survival and growth (Deb and Temjensangba, 2005, 2006b, 2007b; Mukherjee, 1983; Kumaria and Tandon, 1994; Temjensangba and Deb, 2005a). The acclimatized plants were transferred to Departmental botanical garden and the performances of these transplanted plants were monitored at regular interval.

In the current investigation, the plantlets in the hardening condition were found to develop new roots. These newly developed roots get attached to the support medium with the passage of time and vigorous growth of the plantlets were observed. The roots attached themselves mostly to the charcoal pieces and moss, which strongly suggest the suitability of the material for the purpose. In other words, it resembles the velamenous root getting attached to the branch or the trunk of tree in natural habitat. It was also observed that the transplanted regenerates were dark green and healthy with emergence of new roots and leaves just after one month of transfer in the potting mix.

Conclusion

During the present investigation protocols were established for culture initiation from aerial roots, immature seeds/embryos of various developmental stages, foliar explants and nodal explants of *Cymbidium aloifolium* and *Cymbidium iridioides*, regeneration of plantlets and mass multiplication. These techniques open new routes for

in vitro mass multiplication of these two economically important orchids of North-East India in general and Nagaland in particular. The protocols established for culture initiation from aerial roots, foliar explants and nodal explants of both the species indicates the possibility of using alternative explants and demands further research to exploit these explants sources. The protocols may be used by the local commercial orchid growers, NGOs and Government agencies associated with floriculture, which will add to economic development of Nagaland. The present investigation also offers a newer possibility of using the low costs raw materials like foam, coconut coir, betel-nut coir, leaf litter as alternatives to agar which will reduce the production cost considerably and will help in popularizing the plant tissue culture technique. Works are in progress with considerable success to use these substratums in non-orchid species.

Chapter – 5

Summary

North-East India is one of the richest zones with biological values. It is blessed with rich and varied heritage of natural biotic resources including orchids. There are about 780 species of orchids in North-East India and the Eastern Himalayas out of which Nagaland is endowed with 396 species from 92 genera. But, these valuable orchid resources are under threat and are facing the grim possibility of extinction without realising their potential due to various anthropogenic activities. Hence, there is an imperative need for both conservation and sustainable utilization of orchids. The present investigation was undertaken with an objective for rapid mass multiplication and conservation of two economically important orchid species viz., *Cymbidium aloifolium* (L.) and *Cymbidium iridioides* D. Don of North-East India. The cultures were initiated from different explants sources like immature embryos/seeds, foliar explants, aerial roots and nodal explants of the two selected species.

In both the species cultures were initiated from immature embryos/green pods of different developmental stages. In *C. aloifolium,* the seed cultured from green pod of 9 months after pollination (MAP) resulted optimum germination while, in *C. iridioides,* seeds of 10 MAP were found suitable for non-symbiotic seed germination. Seeds from <8 and <6 MAP old pods of *C. aloifolium* and *C. iridioides* respectively either failed to

germinate or showed delayed response and supported poor germination. On the other hand the older seeds of both the species showed a decline in germination rate. In both the species, the first sign of germination was noted as nodular swelling of seeds after 25 days of culture in *C. aloifolium* and 20 days of culture in *C. iridioides*.

About 90% seed germination in *C. aloifolium* was achieved on MS medium followed by Mitra *et al* medium (70%) containing sucrose (2%) (w/v). While in *C. iridioides*, optimum seed germination (95%) was registered on MS medium followed by Mitra *et al* medium (65%) containing sucrose (2%) (w/v). Amongst the different quality and quantity of organic carbon sources used, sucrose at a concentration of 2% supported optimum germination in both the species. Both dextrose and glucose could not support healthy germination. Amongst the different plant growth regulators (PGRs) used in different combinations, in *C. aloifolium*, optimum germination was accomplished on medium containing NAA-BA (3 + 6 μM respectively in combination). But in *C. iridioides* a combination of NAA-BA (3 + 3 μM respectively in combination) was found optimum. With both the species cultures maintained under full light condition (40 μmol m^{-2} s^{-1} light intensity) at 12/12 hr (D/L) photoperiod and at 24±1°C supported better germination followed by diffused light condition (20 μmol m^{-2} s^{-1}).

Apart from agar other materials like betel-nut coir, coconut coir, foam disk and leaf litter could be successfully used with differential success for seed germination. In *C. aloifolium* germination rate was slightly higher (~90%) on media containing foam as substratum compared to agar gelled medium (80%). Apart from higher rate of germination, foam containing medium supported faster germination where within 45 days of culture seeds germinated and formed PLBs. Seeds germinated successfully and formed PLBs on agar gelled medium and media containing betel nut coir, coconut coir,

foam and forest leaf litter as substratums within 58, 49, 43, 45 and 45 days respectively. But in case of *C. iridiouides* agar gelled medium supported better germination (~95%) compared to media containing foam (85%), betel-nut coir (90%), coconut coir (70%) and leaf litter (50%) as substratums. Though germination rate was high on agar gelled medium, it required longer duration compared to other substratums. Besides the time taken for germination and per cent germination, subsequent differentiation was equivalent to that achieved on agar gelled medium and in some cases the alternative substratum out performed agar. Amongst the different substratums studied in the present investigation, better germination and subsequent differentiation was registered on medium containing 'Foam' followed by 'Coconut coir, 'Betel-nut coir' and 'Leaf litter'.

In the current investigation cultures were also initiated from the foliar explants (~5 wk old), aerial roots (~5-6 wk old) and nodal explants of both the species from *in vitro* source. In *C. aloifolium* meristematic loci invoked after about 65 days of culture initiation where as many as 10 shoot buds/PLBs formed per explants in about 52% of cultures. Amongst the different PGRs tested, optimum response was registered on MS medium containing sucrose (3%), NAA and BA (6 and 9 µM respectively in combination). With *C. iridioides*, ~90% cultures responded positively after 90 days of culture where 20 shoot buds developed at the basal part of the foliar explants on medium containing NAA and BA (9 and 3 µM respectively) in combination.

The regeneration from aerial roots of both the species remained recalcitrant to *in vitro* morphogenetic response in the absence of PGRs. In case of *C. aloifolium*, ~60% explants responded positively after 20 days of culture on MS medium containing sucrose (3%) and KN (3 µM) where shoot buds and PLBs formed at both ends of the root. While, in case of *C. iridioides*, cultured aerial roots remained recalcitrant on different PGRs enriched medium unless activated charcoal (AC) was incorporated in the medium. The

MS medium containing sucrose (3%), AC (0.1%) and IAA (3 μM) supported optimum morphogenetic response (50%) after 40 days of culture.

Sprouting of the shoot buds/PLBs from the nodal region of the segments were initiated in both the species. In about 90% cultures, ~ 5 shoot buds/PLBs developed per explants at a single node within 15 days of culture on MS medium supplemented with sucrose (3%) and NAA (9 μM). Whereas, from the nodal segments of *C. iridioides* optimum response was recorded on MS medium fortified with sucrose (3%) and NAA (3 μM) where as many as 6 shoot buds/PLBs developed from a node after 4 days of culture.

The PLBs/shoot buds formed from non-symbiotic seed germination, foliar explants, aerial roots and nodal segments of both the orchid species were cultured on three different basal media viz., Knudson 'C', Mitra *et al* and MS media with different adjuncts for regeneration of plantlets and mass multiplication. Better growth, development and differentiation into rooted plantlets were recorded on MS basal medium for both the species. Further studies with different strengths of MS medium (0, 1/4th, ½, 3/4th and full strength) were conducted on regeneration of plantlets and culture proliferation and it was found that full strength MS medium supported the healthy regeneration and culture proliferation compared to other strengths. The basal medium was supplemented with different types of organic carbon sources (dextrose, glucose and sucrose at a concentration of 0-4%) and it was found that in both the species optimum regeneration and shoot bud formation were obtained on basal media containing sucrose (3%).

In both the species, though incorporation of CH and CW resulted in healthy plantlets, most of the plantlets were either stunted in growth or etiolated. Addition of CH (100 mgl^{-1}) and CW (15%) singly or CH (100 mgl$^{-1)}$ and CW (10%) in combination in *C. aloifolium* supported the formation of 10 shoot buds per explant and healthy but stunted

plantlets. In case of *C. iridioides*, a synergistic effect of CW and CH on formation of shoot buds and healthy plantlets were achieved on medium containing a combined treatment of CH (100 mgl^{-1}) and CW (15%) where as many as 20 shoot buds per explants were formed and exhibited dark green healthy plantlets.

Different PGRs marked a pronounced effect on regeneration of plantlets and mass multiplication. In *C. aloifolium*, optimum response for plantlet regeneration and mass multiplication was achieved on MS medium containing a lone treatment of BA (3 μM) fortified with sucrose (3%), where as many as 12 shoot buds/PLBs were formed per explant with the first leaf and root sprouting just after 21 days of culture. In case of *C. iridioides* better regeneration and multiplication were obtained on MS medium containing a combined treatment of NAA and BA (3 + 6 μM respectively) supplemented with sucrose (3%), CH (100 mgl^{-1}) and CW (15%), where as many 20 shoot buds/plantlets formed after 3-4 weeks.

The advanced stage PLBs/shoot buds were also maintained on the regeneration medium containing different alternative substratums such as betel-nut coir, coconut coir and foam besides agar. In *C. aloifolium*, amongst the different substratums, better regeneration and multiple shoot bud formation were registered on medium containing foam as the substratum where as many as 10 shoot buds were formed. Fewer shoot buds and smaller plantlets were observed on medium containing coconut coir and betel nut coir as substratum which also took longer duration for the development of leaves and roots. In case of *C. iridioides*, amongst the different substratums used, agar gelled medium and foam incorporated medium supported similar regeneration and culture proliferation compared to the other two substratums supported media. It was observed that the initial response was better on agar gelled medium as cultures establish on this medium faster compared on the other substratums. However, once the cultures establish

themselves on the alternative substratums especially on foam and coconut coir, they exhibited healthier growth and rapid culture proliferation compared to cultures on agar gelled medium.

The well rooted plantlets on the regeneration medium were maintained for ~2-3 passages for further growth followed by transferring them in the hardening medium. The well rooted healthy plantlets of both the species were hardened for 6-7 wk in normal laboratory condition in culture vials containing $1/10^{th}$ strength of MS salt solution supplemented with 1% (w/v) sucrose freed from any PGR. In each culture vial different types of supporting materials such as charcoal pieces, brick pieces, and chopped mosses (at 1:1 ratio) were incorporated before the plantlets are transferred. The hardened plants were then transferred to community potting mix containing sand, charcoal pieces, brick pieces, coconut husk and decayed wood (1:1:1:1 ratio) with a layer of moss which were then covered with holed poly bags. The potted plants were maintained in a shaded place and fed with MS liquid salt solution ($1/10^{th}$ strength) for the initial 2-3 wk. The potted plants were exposed to normal day light for about 1 hr in a day for initial one wk and subsequently increased the exposure period by 2 hr from the second wk and finally after one month the plantlets were left in the normal full day light condition which were kept for about 7-8 wk before transferring to the wild. During this process plantlets turned deep green. In *C. aloifolium* ~80% of the transplants survived to form fully developed plants after two months of potting while in case of *C. iridioides* ~75% transplants survived after two months of transfer. It was also observed that the transplanted regenerates were dark green and healthy and new roots started emerging after one month of transfer in the potting mix.

The protocols developed during the present investigation opens new routes for *in vitro* mass multiplication of these two economically important but over-exploited orchids

of North-East India. The protocols established for culture initiation from aerial roots, foliar explants and nodal explants of both the species indicates the possibility of using alternative explants and demands further research to exploit these explants sources. The protocols may be used by the local commercial orchid growers, NGOs and Government agencies associated with floriculture, which will add to economic development of North-East India. The present investigation also offers a newer possibility of using the low costs raw materials like foam, coconut coir, betel-nut coir, leaf litter as alternatives to agar which will reduce the production cost considerably and will help in popularizing the plant tissue culture technique. Works are in progress with considerable success to use these substratums in non-orchid species.

References

Abdul Karim AG and Hairani H. 1990. Leaf culture of some Malaysian orchids. *In*: *Proc. International Conference and Exhibition on Orchids and Ornamental Plants.* Kuala Lumpur, Malaysia. Pp. 12.

Aggarwal S, Sharma S and Pathak P. 2006. On the utility of coir as a substratum for seed germination in Cymbidium pendulum (Roxb.) Sw.: A study (Abstract). *In*: *8th National Seminar on Orchid Conservation, Improvement and Commercialization. Punjab University, Chandigarh, India.* March 18-20, Pp 39.

Agnihotri S, Singh SK, Jain M, Sharma M, Sharma AK and Chaturvedi HC. 2004. *In vitro* cloning of female and male *Carica papaya* through tips of shoots and inflorescences. *Indian J Biotech,* 3: 235-240.

Arditti J and Ernst R. 1993. Micropropagation of Orchids. *John Wiley and Sons Inc.*, New York.

Arditti J, Clements MA, Fast G, Hadley G, Nishimura G and Ernst R. 1982. Orchid seed germination and seedling culture- A manual. *In*: *Orchid Biology- Reviews and Perspective* Vol.II. (Ed. J. Arditti). Cornell University Press, Ithaca, New York. Pp. 243-370.

Arnold SV and Ericksson T. 1984. Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L.) Karst. *Pl Cell Tiss Org Cult,* 3: 257-264.

Babbar SB and Jain R. 1998. 'Isubgol' as an alternative gelling agent for plant tissue culture media. *Pl Cell Rep*, 17: 318-322.

Babbar SB and Jain R. 2006. Xanthan gum: An economical partial substitute for agar in microbial culture media. *Curr Microbiol*, 52: 287-292.

Babbar SB, Jain R and Walia N. 2005. Guar gum as gelling agent for plant tissue culture media. *In Vitro Cell Dev Biol Plant*, 41: 258-261.

Beechey CN. 1970. Propagation of orchids from aerial roots. *Amer Orchid Soc Bull*, 39: 1085–1088.

Bejoy M, Kumar CS, Radhika BJ and Joemon J. 2004. Asymbiotic seed germination and early seedling development of the Dove orchid *Peristeria elata* Hook. *J Orchid Soc India*, 17: 75-79.

Bhadra SK and Hossain MM. 2003. *In vitro* germination and micropropagation of *Geodorum densiflorum* (Lam.) Schltr., an endangered orchid species. *Plant Tissue Cult*, 13: 165-171.

Bhadra SK and Hossain MM. 2004. Induction of embryogenesis and direct organogenesis in *Micropera pallida* Lindl., an epiphytic orchid of Bangladesh. *J Orchid Soc India*, 18: 5-9.

Calvo NR. 1993. Evolutionary demography of orchids: Intensity and frequency of population of pollinators and cost of fruiting. *Ecology*, 74: 1033-41.

Chadha KL. 1992. The Indian orchid scenario. *J Orchid Soc India*, 6: 1-4.

Chen TY, Chen JT and Chang WC. 2004. Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. *Plant Cell Tiss Org Cult*, 76: 11-15.

Chowdhery HJ. 1998. Orchid flora of Arunachal Pradesh. Bishen Singh Mahendra Pal Singh, Dehradun. India.

Chowdhery HJ. 2001. Orchid diversity of northeast India. *J Orchid Soc India*, 15: 1-17.

Chaturvedi HC and Sharma AK. 1986. Mericloning of orchids through culture of tips of leaves and roots. *In*: *Biology, Conservation and Culture of Orchids.* (Ed. SP Vij) Affiliated East-West Press, New Delhi. Pp. 469-472.

Deb CR. 2010. Orchids and sustainable economic development of Nagaland. *In*: Proc. of National Conference on "*Orchids: Systematics and Diversity Analysis for Conservation and Sustainable Utilization*", GBPIHED, Almora, March 19-21. Pp. 61-62.

Deb CR and Imchen T. 2008. Orchid Diversity of Nagaland. *SciChem Publishing House*, Udaipur, Rajasthan, India.

Deb CR and Imchen T. 2010. An efficient *in vitro* hardening technique of tissue culture raised plants. *Biotechnology*, 9(1): 79-83.

Deb CR and Sungkumlong. 2008. *In vitro* regeneration and mass multiplication of *Taenia latifolia* (Lindl.) using immature seeds: a threatened terrestrial orchid. *J Pl Biol*, 35: 1-6.

Deb CR and Sungkumlong. 2009. Rapid multiplication and induction of early *in vitro* flowering in *Dendrobium primulinum* Lindl. *J Pl Biochem and Biotech*, 18: 241-244.

Deb CR and Sungkumlong. 2010. Regenerative competence of foliar explants of *Coelogyne suaveolens* and *Taenia latifolia*: two threatened orchids of North-East India. *Appl Biol Res*, 12: 1-9.

Deb CR and Tandon P. 2004. Factors influencing initiation of embryogenic cultures in *Pinus kesiya* Royle ex. Gord. *Indian J Biotech*, 3: 589-593.

Deb CR and Temjensangba. 2005. *In vitro* regenerative competence of *Cleisostoma racemiferum* (Orchidaceae) aerial roots. *J Pl Biochem Biotech*, 14: 35-38.

Deb CR and Temjensangba. 2006a. On the regeneration potential of *Arachnis labrosa* (Lindl. Ex. Paxt.) Reichb. root segments: A study *in vitro*. *Phytomorphology*, 56: 79-83.

Deb CR and Temjensangba. 2006b. *In vitro* propagation of the threatened terrestrial orchid, *Malaxis khasiana* Soland ex. Swartz through immature seed culture. *Indian J Exp Biol*, 44: 762-766.

Deb CR and Temjensangba. 2007a. Direct regeneration of shoot buds in *Arachnis labrosa* foliar explants. *J Orchid Soc India*, 21: 7-9.

Deb CR and Temjensangba. 2007b. Rapid mass multiplication of *Cleisostoma racemiferum* (Lindl.) Garay: An endangered orchid. *J Pl Biol,* 34: 99-105.

Deb CR, Jamir NS and Temjensangba. 2003. Orchid Diversity of Nagaland - A Revised Status. *J Orchid Soc India*, 17: 5-15.

Deb CR, Deb MS, Jamir NS and Imchen T. 2009. Orchids in indigenous system of medicine in Nagaland, India. *Pleione*, 3(2): 209-211.

Deberg PC. 1983. Effect of agar brand and concentration on the tissue culture media. *Physiol Plant*, 59: 270-276.

Devi J, Nath M, Devi M and Deka PC. 1990. Effect of different media on germination and growth of some North East Indian species of *Dendrobium*. *J Orchid Soc India*, 4: 45-49.

Devi CG, Damayanti M and Sharma GJ. 1998. Aseptic embryo culture of *Vanda coerulea* Grief. *J Orchid Soc India*, 12: 83-87.

FSI. 2000. State of Forest Report, India.

Gamborg OL, Miller RA and Ojima K. 1968. Nutrient requirements of suspension cultures of soyabean root cells. *Exp Cell Res*, 50:151-158.

George EF. 1993. Plant propagation by tissue culture. *Part I: The Technology Edington: Exegetics Ltd*, 3-36.

George PS and Ravishankar GA. 1997. *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. *Plant Cell Rep*, 16: 490-494.

George EF and Sherrington PD. 1984. Plant propagation by tissue culture: Handbook and Directory of Commercial Laboratories. *Exegetics*, Hants.

Gill NK, Gill R and Gosal SS. 2004. Factors enhancing somatic embryogenesis and plant regeneration in sugarcane (*Saccharum offinarum* L.). *Indian J Biotech,* 3: 119-123.

Gupta PK and Grob JA. 1995. Somatic embryogenesis in conifers. *In*: *Somatic embryogenesis in woody plants.* (Eds. V Jain, P Gupta and R Newton), Kluwer Academic Publishers, The Netherlands. Pp. 81-98.

Hadley G. 1970. The interaction of kinetin, auxin and other factors in the development of north temperate orchids. *New Phytol*, 69: 549-555.

Hadley G and Harvais G. 1968. The effect of certain growth substances on asymbiotic germination and development of *Orchis purpurella*. *New Phytol*, 67: 441-445.

Harley JL. 1959. The biology of Mycorrhiza. *Leonard Hill*, London. Pp. 233.

Hegde SN and Sinha SK. 2002. Conservation of rare and endangered vandaceous orchids adopting biotechnological approaches. *In*: *Role of plant tissue culture in biodiversity conservation and economic development.* (Eds. SK Nandi, LMS Palni and A Kumar), Gyanodaya Prakashan, Nainital. India. Pp 521-530.

Henderson WE and Kinnersley AM. 1988. Corn starch as an alternative gelling agent for plant tissue culture media. *Plant Cell Tiss Org Cult*, 15: 17-22.

Hynniewta TM, Kataki SK and Wadhwa BM. 2000. Orchid of Nagaland. Botanical Survey of India, Calcutta.

Ishii Y, Takamura T, Goi M and Tanaka M. 1998. Callus induction and somatic embryogenesis of *Phalaenopsis*. *Plant Cell Rep*, 17: 446-450.

Islam MO, Matsui S and Ichihashi S. 2003. Effect of light intensity and quality on callus growth and photosynthetic pigments content in *Phalaenopsis*. *J Orchid Soc India*, 17: 79-86.

IUCN. 2000. IUCN Red list of threatened Species, Switzerland: The World Conservation Union.

Jain N and Babbar SB. 2002. Gum katira – a cheap gelling agent for plant tissue culture media. *Plant Cell Tiss Org Cult*, 71: 223-229.

Jain N, Gupta S and Babbar SB. 1997. Isubgol as an alternative gelling agent for plant tissue culture media. *J Pl Biochem Biotech*, 6: 129-131.

Jain N, Anjaiah V and Babbar SB. 2005. Guar gum: a cheap substitute for agar in microbial culture media. *Lett Appl Microbiol*, 41: 345-349.

Johansson LB. 1988. Increased induction of embryogenesis and regeneration in anther cultures of *Solanum tuberosum* L. *Potato Res*, 31: 145-149.

Johnson TR, Stewart SL, Dutra D, Kane ME and Richardson L. 2007. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)-preliminary evidence for the symbiotic culture advantage. *Plant Cell Tiss Org Cult*, 90: 313-323.

Kalimuthu K, Senthilkumar R and Vijayakumar S. 2007. *In vitro* micropropagation of orchid, *Oncidium* sp. (Dancing dolls). *African J Biotech*, 6: 1171-1174.

Kannan N. 2009. An *in vitro* study on micropropagation of *Cymbidium* orchids. *Curr Botanica*, 3: 244-250.

Kataki SK, 1986. Orchids of Meghalaya. *Forest Department*, Shillong, Meghalaya.

Katiyar RS, Sharma GD and Mishra RR. 1987. Asymbiotic seed germination and seedling development in *Coelogyne punctulata* and *Aerides multiflorum*. *Indian Forester*, 113: 574-577.

Kaur P and Vij SP. 1995. Morphogenetic response of *Rhynchostylis retusa* Bl. Inflorescence segment. *J Orchid Soc India*, 9: 85-90.

Kaur S and Vij SP. 2000. Regeneration potential of *Saccolabium papillosum* leaf segments. *J Orchid Soc India*, 14: 67-73.

Kaur S and Bhutani KK. 2009. *In vitro* propagation of *Vanda testacea* (Lindl.) reichb.f.-a rare orchid of high medicinal value. *Plant Tiss Cult and Biotech*, 19: 1-7.

King G and Pantling P. 1898. The orchids of Sikkim Himalaya. *Ann Roy Bot Gard*, Calcutta, 8: 1- 342.

Knudson L. 1922. Non-symbiotic germination of orchid seeds. *Bot Gaz*, 73: 1-25.

Knudson L. 1946. A new nutrient solution for germination of orchid seeds. *Amer Orchid Soc Bull*, 15: 214-217.

Kohlenbach HW and Wernicke W. 1978. Investigations on the inhibitory effect of agar and the function of active carbon in anther culture. *Z Pflanzenphysiol*, 86: 463-472.

Kosir P, Skof S and Luthar Z. 2004. Direct shoot regeneration from nodes of *Phalaenopsis* orchids. *Acta Agriculturae Slovenica*, 83: 233-242.

Kumar SC, Manilal KS. 1994. A catalogue of Indian Orchids. Bishen Singh Mahendra Pal Singh, Dehradun.

Kumaria S and Tandon P. 1994. Clonal propagation and establishment of plantlets of *Dendrobium fimbriatum* var. *oculatum* Hook.f. *In*: *Advances in Plant Tissue Culture in India* (Ed. P Tandon). Pragati Prakashan, Meerut. India, 218-231.

Laishram JM and Devi S, Y. 1999. Micropropagation of *Renanthera imschootiana* Rolfe through shoot tip, axillary bud and leaf segment cultures. *J Orchid Soc India*, 13: 1-4.

Lal N, Ahuja PS, Kukreja AK and Pandey B. 1988. Clonal propagation of *Picrorhiza kurroa* Royle ex Benth. by shoot tip culture. *Plant Cell Rep*, 7: 202-205.

Lavanya M, Venkateshwarlu B and Devi BP. 2009. Acclimatization of neem microshoots adaptable to semi-sterile conditions. *Indian J Biotech*, 8: 218-222.

Lee P, Loh CS and Goh CJ. 1995. An *in vitro* method for rapid propagation of monopodial hybrid Aranda 'Deborah' using thin section culture. *Plant Cell Rep*, 14: 60-65.

Leetham DS. 1974. Regulation of cell division in plant tissues by the cytokinin of coconut milk. *Plant Physiol*, 32: 66-67.

Li Z and Xu L. 2009. *In vitro* propagation of white-flowered mutant of *Rhynchostylis gigantean* (Lindl.) Ridl. Through immature seed-derived protocorm-like bodies. *J Hortic For*, 1: 93-97.

Martin KP. 2007. Micropropagation of the bamboo orchid (*Arundina graminifolia* (D.Don) Hochr.) through protocorm-like bodies using node explants. *Prop Ornamen Pl*, 7: 97-100.

Mathews VH and Rao PS. 1985. *In vitro* culture of Vanda hybrid (Vanda TMA X Vanda Miss. Joaquim) 1. Studies on protocorm explants. *In*: *Proc. Indian Natl Sci Acad*, 51: 96-103.

Mc Kendrick S. 2000. *In vitro* germination of Orchids: a manual. Ceiba Foundation for Tropical Conservation.

Mc Lachlan. 1985. Macroalgae (seaweeds): industrial resources and their utilization. *Plant Soil*, 89: 137-157.

Mitra GC. 1986. *In vitro* culture of orchid seeds for obtaining seedling. *In*: *Biology, Conservation and culture of orchids* (Ed., SP Vij), Affiliated East-West Press. New Delhi, India. Pp. 401-412.

Mitra GC, Prasad RN and Roy Chowdhury A. 1976. Inorganic salt and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. *Indian J Exp Biol*, 14: 350-351.

MoEF. 1999. National Policy and Macrolevel Action Strategy on Biodiversity. *Ministry of Environment and Forests*, Government of India, New Delhi, Pp. 74.

Morel G. 1960. Producing virus-free *Cymbidiums*. *Amer Orchid Soc Bull*, 29: 495-497.

Mukherjee SK. 1983. Orchids. Published by P.C. Bedi, Under Secretary, for the *Indian Council of Agricultural Research*, New Delhi.

Murashige T. 1974. Plant propagation through tissue culture. *Ann Rev Pl Physiol*, 25: 135-166.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plantarum*, 15: 473-497.

Myers N. 1990. The biodiversity challenge, expanded host spots analysis. *The Environmentalist*, 10: 1-14.

Nagaraju V and Upadhyaya RC. 2001. *In vitro* morphogenetic response of *Cymbidium* PLBs to three basal media and activated charcoal. *J Orchid Soc India*, 15: 59-64.

Nagaraju V, Das SP, Bhutia PC and Upadhyaya RC. 2004. *In vitro* multiplication of *Dendrobium chrysotoxum* and two Dendrobium crosses (*D. nobile* x *D. nobile* var. *alba* and *D. nobile* x *D. heterocarpum*) through embryo culture. *J Orchid Soc India*, 18: 47-51.

Nath M, Devi J, Borthakur B, Sharma J and Deka PC. 1991. Embryo culture of *Rhynchostylis retusa* and *Vanda coerulea*. *J Orchid Soc India,* 5: 97-101.

Nayak NR, Patnaik S and Rath SP. 1997. Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter & McCann. *Plant Cell Rep*, 16: 583-586.

Nene YL, Shiela VK and Moss JP. 1996. Tapioca-a potential substitute for agar in plant tissue culture media. *Curr Sci*, 70: 493-494.

Nitsch JP and Nitsch C. 1969. Haploid plants from pollen grains. *Science*, 163: 85-87.

Pasqualetto PL, Zimmerman RH and Fordham IM. 1988. The influence of cation and gelling agent concentrations on vitrification of apple cultivars *in vitro*. *Plant Cell Tiss Org Cult*, 14: 31-40.

Pathak P, Mahant KC and Gupta A. 2001. *In vitro* propagation as an aid to conservation and commercialization of Indian Orchids: Seed culture. *In*: *Orchid: Science and Commerce,* (Eds. P Pathak, RN Sehgal, N Shekhar, M Sharma and A Sood). Bishen Singh Mahendra Pal Singh, Dehra Dun, India, Pp. 319-362.

Paul TL. 1999. Tissue culture techniques in *in vitro* plant conservation. *In*: *Plant Conservation Biotechnology.* (Ed., EE Benson). Taylor & Francis Ltd, London, Pp. 2-62.

Peres LEP, Amar S, Kerbauy GB, Salatino A, Zaffari AR. and Mercier H. 1999. Effect of auxin, cytokinin and ethylene treatments on the endogenous ethylene and auxin-to cytokinins ratio related to direct root-tip conversion of *Catasetum fimbriatum* Lindl. (Orchidaceae) into buds. *J Pl Physiol,* 155: 551-555.

Philip VJ and Nainar SAZ. 1988. *In vitro* transformation of root meristem to shoot and plantlets in *vanilla planifolia*. *Ann Bot*, 61: 143-149.

Pradhan UC. 1979. Indian orchids- Guide to identification and culture (Vol. II), Primulaceae Books. Kalimpong, West Bengal, India.

Prasad AB, Latha PG and Seeni S. 2000. Micropropagation of terrestrial orchids *Anoeetochilus sikkimensis* and *A. regalis*. *Indian J Exp Biol*, 38: 149-154.

Raghuvanshi AN, Mishra RR and Sharma GD. 1986. Effect of *p*H on asymbiotic seed germination and seedling development of orchids. *In*: *Biology, conservation and culture of orchids.* (Ed., SP Vij), Affiliated East-West Press, New Delhi, India. Pp. 453-462.

Raghuvanshi AN, Mishra RR, and Sharma GD. 1991. Symbiotic seed germination and seedling development of orchids at different temperatures. *J Orchid Soc India*, 5: 61-69.

Rao AN, Ahmed N and Hegde SN. 1998. Culture notes from Tipi-4: *In vitro* culture of some rare and endangered orchids of Arunachal Pradesh. *In*: *International Festival of Orchids*. Orchid Society of Arunachal. Pp. 16-27.

Rotor G. 1949. A method of vegetative propagation of *Phalaenopsis* species and hybrids. *Amer Orchid Soc Bull*, 18: 738-739.

Roy J. and Banerjee N. 2003. Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. Var. *oculatum* HK. F. *Scientia Hortic*, 97: 333-340.

Sagawa Y. 1963. Green pod culture. *The Florida Orchidist*, 6: 296-297.

Sauleda RP. 1976. Harvesting time for orchid seed capsule for the green pod culture process. *Amer Orchid Soc Bull*, 45: 305-308.

Schenk RU and Hildebrandt AC. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can J Bot*, 50: 199-204.

Scheurich P, Schnabl H, Zimmermann U and Klein J. 1980. Immobilisation and mechanical support of individual protoplasts. *Biochim Biophys Acta,* 598: 645-651.

Seeni S. 1988. Micropropagation of blue Vanda using leaf bases. *In*: *Proceedings of the national seminar on current Research Trends in Indian Orchids with special reference to tissue culture technology* (Ed., SP Vij). *The Orchid Society of India,* Chandigarh, Pp. 22.

Seeni S and Latha PG. 1992. Foliar regeneration of endangered red Vanda *Renanthera imschootiana* Rolfe (Orchidaceae). *Plant Cell Tiss Org Cult,* 29: 167-172.

Sharma SK and Tandon P. 1986. Influence of growth regulator on asymbiotic germination and early seedling development of *Coelogyne punctulata* lindl. *In*: *Biology, conservation and culture of orchids* (Ed., SP Vij). Affiliated East-West Press, New Delhi. Pp. 441-451.

Sharma SK and Tandon P. 1990. Asymbiotic germination and seedling growth of *Cymbidium elegans* Lindl. and *Coelogyne punctulata* Lindl. as influenced by different carbon sources. *J Orchid Soc India*, 12: 83-87.

Sheela VL, Rajmohan K, Anita S and Sarada S. 2004. Effect of growth regulators on the develoment and multiplication of protocorm like bodies in Dendrodium cv. sonia. *J orchid Soc India,* 18: 21-23.

Sheelavanthmath SS, Murthy HN, Pyati AN, Ashok Kumar HG and Ravi Shankar BV. 2000. *In vitro* propagation of endangered orchid *Geodorum densiflorum* through rhizome section culture. *Plant Cell Tiss Org Cult,* 60: 151-154.

Shiau YJ, Nalawade SM, Hsia CN, Mulabagal V and Tsay HS. 2005. *In vitro* propagation of the Chinese medicinal plant, *Dendrobium candidum* Wall. Ex Lindl., from axenic nodal segments. *In Vitro Cell Dev Biol Pl*, 41: 666-670.

Singh F and Prakash D. 1984. *In vitro* propagation of *Thunia alba* (wall.) Reichb.f. through flower stalk cuttings. *Sci Hortic,* 24: 385-390.

Singha S. 1984. Influence of two commercial agars on *in vitro* shoot proliferation of 'Almey' crabapple and 'Seckel' pear. *Hort Sci*, 19: 227-228.

Sinha SK and Hegde SN. 1997. Regenerative competence of *Aerides rosea* Loddiges Ex Paxt. from axillary bud: A study *in vitro*. *Arunachal Forest News*, 15: 47-50.

Sinha SK and Hegde SN. 1999. Regeneration of plantlets from *in vitro* leaf culture of Renades Arunoday Hybrid (*Aerides rosea* Loddiges Ex. Paxt. X *Renanthera imschootiana* Rolfe). *J Orchid Soc India*, 13: 19-24.

Sinha SK, Singh LS and Hegde SN. 1998. *In vitro* multiplication of *Aerides rosea* Loddiges ex. Paxt. through asymbiotic seed germination. *Arunachal For News*, 16: 38-44.

Sinha SK, Singh LS and Hegde SN. 2002. Effect of nutrient concentrations on *in vitro* growth and differentiation of protocorm like bodies of Renades Arunodaya. *In*: *Role of plant tissue culture in biodiversity conservation and economic development*. (Eds., SK Nandi, LMS Palni and A Kumar). Gyanodaya, Nainital, India. Pp. 229-236.

Sood A and Vij SP. 1986. *In vitro* root segment culture of *Rhynchostylis retusa* Bl. *In*: *Biology, Conservation and Culture of Orchids* (Ed., SP Vij) Affiliated East-West Press, New Delhi, India. Pp. 463-468.

Stewart SL and Kane ME. 2006. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tiss Org Cult*, 86: 147-158.

Sungkumlong and Deb CR. 2008. Effects of different factors on immature embryo culture, PLBs differentiation and rapid mass multiplication of *Coelogyne suaveolens* (Lindl.) Hook. *Indian J Exp Biol*, 46: 243-248.

Sungkumlong and Deb CR. 2009. Regeneration competence of *Taenia latifolia* (Lindl.) ex Hook pseudobulb segments: an *in vitro* study. *Indian J Biotech*, 8: 121-126.

Sunitibala H and Kishor R. 2009. Micropropagation of Dendrobium transparens L. from axenic pseudobulb segments. *Indian J Biotech*, 8: 448-452.

Takhtajan A. 1969. Flowering plants: Origin and dispersal. *Oliver and Boyd*, Edinburgh, U. K.

Talukdar A. 2001. Multiple shoots induction in *Dendrobium aphyllum* Roxb. *J Orchid Soc India*, 15: 35-38.

Tandon P, Rathore TS and Dang JC. 1990. Mass multiplication and conservation of some threatened plant species of Northeast India through tissue culture (Abstract). *In*: VIIth International Congress on Plant Tissue Organ Culture, Amsterndam, Netherlands: IAPTC. Pp. 136.

Temjensangba and Deb CR. 2005a. Regeneration and Mass Multiplication of *Arachnis Labrosa* (Lindl.ex Paxt.) Reichb: A Rare and Threatened Orchid. *Curr Sci*, 88(12): 1966-1969.

Temjensangba and Deb CR. 2005b. Regeneration of plantlets from *in vitro* raised leaf explants of *Cleisostoma racemiferum* Lindl. *Indian J Exp Biol,* 43: 377-381.

Temjensangba and Deb CR. 2005c. Factors Regulating Non-Symbiotic Seed Germination of Some Rare Orchids of Nagaland. *Nagaland Univ Res J,* 3: 48-54.

Temjensangba and Deb CR. 2006. Effect of different factors on non-symbiotic seed germination, formation of protocorm –like bodies and plantlet morphology of *Cleisostoma racemiferum* (Lindl.) Garay. *Indian J Biotech*, 5:223-228.

Thomale H. 1957. Die Orchideen. *Eugen Ulmer Verlag*. Stuttgart.

Tisserat B and Jones D. 1999. Clonal propagation of orchids. *In*: *Plant Cell Culture Protocols: Methods in Molecular Biology, III.* (Ed., RD Hall), Humana Press Inc., Totowa, NJ, USA. Pp. 127-134.

Tsuchiya I. 1954. Possibility of germination of orchids seeds from immature fruits. *Natl Pua Okika O Hawaii Nei*, 4: 11-16.

Vacin EF and Went FW. 1949. Some *p*H changes in nutrient solutions. *Bot Gaz*, 110: 605-613.

Valmayor HL and Sagawa Y. 1967. Ovule culture in some orchids. *Amer Orchid Soc Bull*, 36: 766-769.

Vij SP. 1993. Regeneration response of orchid roots: A study *in vitro*. *J Orchid Soc India*, 7: 61-73.

Vij SP. 1995. Genetic Resources of Orchids. *In*: *Advances in horticulture, Vol. 12, Ornamental plants* (Eds., KL Chadha and SK Bhattacharjee). I. Malhotra Publishing House, New Delhi, India. Pp. 153-181.

Vij SP. 2002. Orchids and tissue culture: Current status. In: *Role of plant tissue culture in biodiversity conservation and economic development*. (Eds., SK Nandi, LMS Palni and A Kumar). Nainital: Gyanodaya Prakash. Pp. 491-502.

Vij SP and Pathak P. 1988. Asymbiotic germination of the saprophytic orchid, *Cymbidium macrorhizon*: A study *in vitro*. *J Orchid Soc India*, 4: 69-88.

Vij SP and Pathak P. 1990. Micropropagation of orchids through leaf segments. *J Orchid Soc India*, 4: 69-88.

Vij SP and Pathak P. 1999. Micropropagation of *Dendrobium moschatum* Wall. through pseudobulb segments. *J Orchid Soc India*, 3: 25-28.

Vij SP and Sharma M. 1997. A Micropropagation system for Vanda Kasem's Delight Tom Boykin. *In*: *Proc. Natl. Seminar on Development Biology and Commercialisation of Orchids and Orchid Show*. Gangtok, Sikkim. Pp. 85.

Vij SP and Aggarwal S. 2003. Regenerative competence of foliar explants: *Vanda Coerulea* Griff. *J Orchid Soc India*, 17: 73- 78.

Vij SP, Sood A and Plaha KK. 1984. Propagation of *Rhynchostylis retusa* Bl. (Orchidaceae) by direct organogenesis from leaf segment cultures. *Bot Gaz*, 145(2): 210-214.

Vij SP, Sood A and Pathak P. 1989. On the utility of rhizome segments in micropropagating *Eulophia hormusjii* Duth. *J Orchid Soc India*, 3: 41- 45.

Vij SP, Sharma V and Kaur S. 1994. Foliar explants and orchid Micropropagation: Vanda Kasem's Delight 'Tom Boykin'. *J Orchid Soc India*, 8: 79-83.

Vij SP, Pathak P and Mahant KC. 1995. Green pod culture of a therapeutically important species *Dactylorhiza hatagirea* (D. Don) Soo. *J Orchid Soc India*, 9: 7-12.

Vij SP, Pathak P and Kher A. 1997. Regeneration response of *Rhynchostylis gigantea* inflorescence segments: a study *in vitro*. *J Orchid Soc India*, 11: 75-78.

Vij SP, Kher A and Pathak P. 2000. Regeneration competence of *Bulbophyllum careyanum* (Hook.) Spreng. Pseudobulb segments. *J Orchid Soc India*, 14: 47-55.

Vij SP, Aggarwal S and Promila Pathak. 2004. Regeneration competence of Cymbidium Great Waltz x valley flower roots: A study *in vitro*. *J Orchid Soc India*, 18: 109-115.

Von Arnold S. 1987. Improved efficiency of somatic embryogenesis in mature embryos of *Picea abies* (L.) Karst. *J Pl Physiol*, 128: 233-244.

Wilson EO. 1988. Biodiversity. National Academy Press, Washington, DC, USA.

Wimber DE. 1963. Clonal multiplication of *cymbidiums* through tissue culture of the shoot meristem. *Amer Orchid Soc Bull*, 32: 105-107.

Wimber DE. 1965. Additional observations on clonal multiplication of *Cymbidium* through culture of shoot meristems. *Cymbidium Soc News*, 20: 7-10.

Wood A, Stedman-Edwards P and Mang J. 2000. The root causes of Biodiversity loss. UK: *World Wide Fund for Nature*. Earth Scan Publications. Pp. 399.

Yam TW and Weatherhead MA. 1988. Germination and seedling development of some Hong Kong orchids. *Lindleya*, 3: 156-160.

Yam TW and MA Weatherhead. 1991. Leaf tip culture of several native orchids of Hong Kong. *Lindleyana*, 6: 147-50.

Yasugi S. 1984. Shortening the period from pollination to getting seedlings by ovule or ovary culture in *Doritis pulcherima* (Orchidaceae). *J Japan Soc Hortic Sci*, 53: 52-58.

Zimmermann RH, Bhardwaj SV and Fordham IM. 1995. Use of starch-gelled medium for plant tissue culture of some fruit crops. *Plant Cell Tiss Org Cult*, 43: 207-213.

List of Publications

Published as Abstract

2. **Pongener A. and Deb CR. 2010**. Immature embryo culture and plant regeneration of *Cymbidium iridioides* D. Don: The role of different factors. *In*: National Conference on "Orchids: Systematics and Diversity Analysis for Conservation and Sustainable Utilization", GBPIHED, Almora, March 19-21. Pp. 61.

1. **Pongener A and Deb CR. 2009.** Use of different substratum as alternative to agar for asymbiotic seed germination and plantlet regeneration of *Cymbidium iridiodes* D. Don: A study *in vitro*. *In:* 96th Session of Indian Science Congress, NEHU, Shillong, January 3-7. **(Section: New Biology; Abstract No: 38), Pp. 32-33.**

Published/Accepted/Communicated in Peer Reviewed Journals

4. **Pongener A and Deb CR.** *In vitro* mass multiplication of *Cymbidium aloifolium* using immature embryos as explant. ***J Pl Biochem Biotech*** (Communicated).

3. **Deb CR and Pongener A**. Use of Different Substratum as Alternative to Agar for Asymbiotic Seed Germination and Plantlet Regeneration of *Cymbidium iridioides* D. Don: A Study *In Vitro*. ***In vitro Cell Dev Biol Pl*** (In-Press).

2. **Deb CR and Pongener A. 2010.** Search of alternative substratum for agar in plant tissue culture. ***Curr Sci***, 98(1): 99-102.

1. **Pongener A. and Deb CR. 2009**. Asymbiotic Culture of Immature Embryos, Mass Multiplication of *Cymbidium iridioides* D. Don. And the Role of Different Factors. ***Intl J Pharma Bio Sci.***, 1(1): 1-14.